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The Roles of Biogenic Amines and Dopamine Receptors in Envenomation by the Parasitoid Wasp *Ampulex compressa*

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I would like to dedicate this dissertation to my fiancée Candice Stafford for her unending support during the light of day, and the days of darkness.

Per Aspera Ad Astra

ABSTRACT OF THE DISSERTATION

The Roles of Biogenic Amines and Dopamine Receptors in Envenomation by the Parasitoid Wasp *Ampulex compressa*

by

Christopher Neil Banks

Doctor of Philosophy
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Dr. Michael E. Adams, Chairperson

The parasitoid wasp *Ampulex compressa* uses a unique biochemical strategy to exploit the American cockroach *Periplaneta americana* as a food source for its offspring. The wasp injects venom directly into the brain and subesophageal ganglion of the cockroach, which leads to a bout of grooming which lasts approximately 30 minutes. The cockroach subsequently falls into a state of hypokinesia, which is characterized by reduction of escape responses and inability to generate spontaneous movements. We investigated potential mechanisms of action for these venom-induced behaviors, focusing predominantly on the possible roles of the biogenic amine dopamine. Reserpine injection produces similar behavioral phenotypes to stung cockroaches by depleting presynaptic stores of biogenic amines. We determined that levels of dopamine, serotonin, octopamine, or tyramine in the head and thoracic ganglia were not depressed in stung cockroaches, but were dramatically reduced in reserpinized animals. We also investigated the effects of *Ampulex* venom and two major venom peptides (DG2847 and

DG2807) on dopamine receptors in relation to sting-induced grooming and hypokinesia induction. The full-length cDNA encoding the D2-like dopamine receptor from *Periplaneta americana* (PeaD2R) was sequenced and cloned into a vector for expression in Chinese Hamster Ovary (CHO-K1) cells. Three dopamine receptors from *Drosophila melanogaster* (dDA1, DAMB, and D2R) were also cloned. Using an aequorin-based cell luminescence assay, pharmacological profiles were generated for each receptor using various synthetic dopamine receptor agonists and antagonists in order to determine the specificity of these compounds on each receptor subtype. The D2-selective compound sulpiride, when preinjected into cockroach hemolymph, significantly reduced sting-induced grooming. Dopamine, a neurotransmitter that has been implicated in sting-induced grooming, was determined to be present in milked venom by high performance liquid chromatography with electrochemical detection. We also investigated functional properties of *Ampulex* venom and venom peptides. We determined that neither *Ampulex* venom, nor the venom peptides, possessed antimicrobial activity, or induced cell death *in vitro*. Milked venom and the venom peptides induced a decrease in ligand-induced luminescence when incubated with CHO-K1 cells. This suggests that calcium mobilization is being altered when exposed to venom, which may disrupt signaling pathways in the central nervous system.

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A Review

Introduction

Parasitoids are organisms that exploit living hosts in order to develop and reproduce. Unlike parasites, parasitoids inevitably end up killing the host, making their relationship with the host more predaceous than parasitic. The insect order Hymenoptera contains the vast majority of insect parasitoids, predominantly wasps. Parasitoid-host interactions and developmental strategies vary greatly from species to species, but in all cases, females must use their natural tools to find a suitable host and exploit it for the sake of their young. In many cases, injection of venom will paralyze the host, rendering it unable to escape oviposition. In others, venom causes behavioral changes that prevent the host from escaping or defending itself from the wasp. Nonetheless, venom injection and subsequent egg-laying and larval development will ultimately result in the death of the host. The focus of my doctoral research was to elucidate the biochemical mechanisms underlying envenomation by the parasitoid wasp *Ampulex compressa*, and determine the molecular components involved.

Wasp Venom Composition and Biological Effects

Animal venoms are complex mixtures of biological substances, including proteins, peptides, polyamines, and low molecular-weight compounds, such as biogenic amines (Abe *et al*. 1989; Blagbrough *et al*. 1994; Hisada *et al*. 2005; Moreau *et al*. 2005). These compounds have a myriad of effects on host animals. Venom toxins are finely tuned to target specific molecules, and can be exploited as pharmacological tools to study cellular processes. Examples include the use of α-neurotoxins (such as α-bungarotoxin

from the Taiwanese banded krait) to isolate and characterize nicotinic acetylcholine receptors (Chang 1999), and tetrodotoxin from the puffer fish to study sodium channels (Narahashi 2008). Venoms are also sources of novel bioactive components which may serve as the foundation for drug discovery. For example, ziconotide is an analgesic derived from ω-conotoxin MVIIA from the cone snail, *Conus magus* (Terlau *et al*. 2004; Molinski *et al*. 2009). Venoms are beginning to receive more attention in the scientific community, as advances in mass spectrometry and genomics are making venom component identification faster and easier (Escoubas *et al*. 2006; Favreau *et al*. 2006; Escoubas *et al*. 2008).

Peptides are major venom components that can have a variety of functions. Antimicrobial venom peptides have been identified from various hymenopteran species, including *Polistes dominulus* (Turillazzi *et al*. 2006), *Anoplius samariensis* (Konno *et al*. 2001), *Vespa bicolor* Fabricius (Chen *et al*. 2008), and *Vespa magnifica* (Xu *et al*. 2006). Bradykinin-like kinins have been identified in two *Vespa* species (Gobbo *et al*. 1995) and in *Megascolia flavifrons* (Piek *et al*. 1984), which have been shown to block synaptic transmission (Piek *et al*. 1987). α- and β-pompilidotoxin (from *Anoplius samariensis* and *Batozonellus maculifrons* respectively) slow the inactivation of Na^+ channels (Sahara *et al*. 2000; Miyawaki *et al*. 2002), and have the ability to distinguish between different types of Na^+ channels (Kinoshita *et al*. 2001). Mastoparan, a tetradecapeptide from wasp venom, is a G-protein activator that is selective for Giα/Goα subunits and competes with receptors for G-protein binding (Higashijima *et al*. 1988). G-protein activation causes

mast cells to release histamine by exocytosis (Aridor *et al*. 1993), which induces pain at the site of venom injection.

Polyamines are low molecular weight molecules that are ubiquitous in cells and are involved in important biological functions. Polyamine toxins found in animal venoms are similar in structure, and are comprised of a polyamine backbone, with a primary amino group or guanidine group on one end, and an aromatic head at the other end (Stromgaard *et al*. 2005). Endogenous polyamines, like spermine and spermidine have been shown to block inward rectifying K^+ channels, contribute to the rectification and intrinsic gating of these ion channels, and block glutamate receptors, particularly N-methyl-D-aspartate (NMDA) receptors (Williams 1997). Polyamine activity has also been observed at nicotinic acetylcholine receptors (nAChRs) as well (Mellor *et al*. 2004). Polyamine toxins from wasps (such as philanthotoxin-433 from the digger wasp *Philanthus triangulum*) and spiders (such as α-agatoxins from *Agelenopsis aperta* and argiotoxin-636 from *Argiope trifasciata*) act in a similar fashion at ionotropic glutamate receptors (Adams 2004; Mellor *et al.* 2004). These toxins are noncompetitive receptor antagonists that are open channel blockers, meaning they bind only after the channel has been activated. However, there is evidence that polyamine toxins antagonize channels in the closed state as well (Jayaraman *et al.* 1999; Mellor *et al.* 2004). Philanthotoxin-433 and its synthetic analogues are the most potent venom-derived polyamines that antagonize nAChRs (Stromgaard *et al.* 2005).

Other compounds with low molecular weights are also present in hymenopteran venoms. Venom from the solitary wasp *Anoplius samariensis* contains γ-aminobutyric

acid (GABA) and glutamic acid, although the physiological functions of these components have not been demonstrated (Hisada *et al.* 2005). Hornets of the genus *Vespa* contain many inhibitory neurotransmitters, including glutamate, GABA, and glycine, in addition to other amino acids, like leucine, arginine, and tryptophan (Abe *et al.* 1989). Honeybee and various types of hornet venom contain dopamine and norepinephrine, which increases heart rate in insects, and may increase the circulation of hemolymph so other venom components can reach their targets more quickly (Owen 1971).

Wasp venoms can disrupt homeostasis in diverse ways and render the victim incapable of defending itself by interfering with the normal biological processes of the host. The venom from the ectoparasitoid wasp *Nasonia vitripennis* (Walker) is a proteinaceous cocktail that induces a number of biological effects in host flies, including depression of respiratory metabolism, increase of lipids in the hemolymph, and developmental arrest which ultimately ends in death (Rivers *et al*. 2006). The venom induces membrane blebbing and cell lysis, most likely due to an increase in sodium ion permeability across the membrane (Rivers *et al*. 2002). The venom also activates phospholipase C, which leads to an increase in intracellular Ca^{+2} from the mitochondria (Rivers *et al*. 2005). Cell death does not occur without Ca^{+2} elevation, because blocking phospholipase C activation prevents Ca^{+2} release from the endoplasmic reticulum, and protects the cell from the lytic effects of the venom (Rivers *et al.* 2005).

The braconid wasp *Cotesia congregata* parasitizes the tobacco hornworm *Manduca sexta* by injecting venom, eggs, and polydnavirus into the hemolymph. This

combination of factors induces the hornworm to enter a state of developmental arrest, where feeding is suppressed and levels of locomotion are decreased. Feeding suppression prevents the hornworm from eating the wasp cocoons, or dislodging the cocoons from the cuticle (Adamo 1998). In this state, the caterpillar cannot metamorphose to the adult stage, and subsequently dies. Although the specific mechanisms behind the developmental arrest are not fully understood, wasp emergence is associated with an accumulation of neuropeptides in the central nervous system (Zitnan *et al*. 1995) and altered levels of developmental hormones and biogenic amines in the hemolymph (Beckage *et al*. 2004; Adamo 2005).

The Digger Wasp, *Liris niger*

Parasitoids use their venom to secure a host for the development of their offspring, but the process of subduing a host can vary for different wasp species. One interesting example is the digger wasp, *Liris niger* Fabr, a solitary wasp that uses crickets as hosts. The digger wasp injects its venom into each of the thoracic ganglia and the subesophageal ganglion (SEG) of the host, which causes immediate total paralysis, which immobilizes the cricket, followed by transient paralysis (Gnatzy 2001). In the transient paralysis phase, the cricket is able to move in response to external stimuli, but lacks the ability to generate movement on its own. Shortly after total paralysis, the wasp physically drags its host to an empty burrow, whereupon an egg is deposited upon it. After a few days, the egg hatches, and the wasp larva feeds on the paralyzed cricket, which remains alive for several days.

The venom of *Liris niger* induces total paralysis by blocking the generation of action potentials, and by inhibiting synaptic transmission. The venom blocks voltage-gated inward sodium currents and increases the threshold for inward current activation, ultimately preventing the initiation of action potentials up to 45 minutes (Ferber *et al*. 2001). This effect is reversible, because washing away the venom leads to complete restoration of action potential generation. In addition, the effects of the venom only act locally at the ganglia which were stung, because no change in neuronal activity was observed in unstung ganglia (Gnatzy 2001). Venom injection also blocks calcium currents in thoracic and abdominal dorsal unpaired median (DUM) neurons, and induced a dramatic increase in leak currents (Ferber *et al.* 2001). Synaptic transmission appears to be compromised as well, as mechanical stimulation of cerci did not evoke any excitatory postsynaptic potentials (EPSPs) in the cockroach giant interneurons. However, application of acetylcholine produced potentials that were identical both in the presence and absence of *Liris* venom (Ferber *et al.* 2001). The combination of these effects efficiently subdues the cricket, allowing the wasp to utilize the animal as food for its larva.

Liris niger venom contains many polypeptides, ranging from 3.5 to 200 kDA in size (Gnatzy *et al*. 2000). The protein content of the venom gland is very similar to the protein content of secreted venom droplets, indicating that the polypeptides found in the venom gland are most likely injected into the ganglia. In contrast, the Dufour's gland has a different peptide fingerprint than the venom reservoir, thus Dufour's gland contents do not contribute as much (if at all) to the venom which is secreted (Gnatzy *et al.* 2000).

The agents responsible for the paralysis and behavioral changes have not yet been identified.

The Emerald Jewel Wasp, *Ampulex compressa*

One of the most striking examples of a parasitoid wasp that manipulates a host for the benefit of its offspring is the solitary ampulicid wasp *Ampulex compressa*. This wasp uses the American cockroach *Periplaneta americana* as a host for its eggs and larvae (Williams 1942). Whereas many parasitoid wasps will directly paralyze their prey, *Ampulex compressa* employs a novel strategy to subdue and manipulate the cockroach. First, the wasp stings directly into the first thoracic ganglion of the cockroach, causing a transient paralysis of the front legs which lasts approximately three minutes. While the prothoracic legs are paralyzed, the wasp performs a second sting directly into the SEG and the brain of the cockroach, causing a dramatic change in its behavior. The cockroach immediately engages in pronounced grooming for approximately thirty minutes, and subsequently falls into a hypokinesic "zombie" state, which lasts 1-2 weeks (Piek *et al*. 1984; Weisel-Eichler *et al*. 1999). The wasp takes advantage of the semi-catatonic cockroach, and walks it to its lair with virtually no resistance. The wasp lays its egg on the cockroach's cuticle, and blocks the opening of the lair with rocks and debris. After a few days, the larva hatches and begins to feed on hemolymph, then crawls into the cockroach, where it directly feeds on the host's tissue and eventually pupates. An adult wasp emerges from the hollowed-out cockroach approximately six weeks later.

There are three characteristic events associated with the sting from *Ampulex compressa*: transient paralysis of the front legs, compulsive grooming, and long-term hypokinesia. Each of these behaviors is a direct consequence of venom injection into the central nervous system and not the result of mechanical irritation, because these behaviors are not observed if the wasp is physically removed from the cockroach before envenomation (Weisel-Eichler *et al*. 1999). Venom extracted from the wasp is also biologically active, and is composed of compounds from the venom gland and Dufour's gland (Moore 2003). Venom can be extracted by placing a female wasp in a modified pipet tip and allowing it to sting through a piece of parafilm. Venom droplets left behind on the parafilm are collected in water and frozen immediately in order to prevent degradation of biologically active compounds. Although droplet volume is quite small, with an average size of 5 nL (Moore 2003), venom collected in this fashion can be used to determine biological functions, as described below.

Transient paralysis of the cockroach's front legs is caused by the first sting into the prothoracic ganglion. This prevents the cockroach from using its front legs for defensive purposes, allowing the wasp to perform the more precise sting into the head ganglia. Extracellular recordings from motor nerve 5r1 of the mesothoracic ganglion (T2) in cockroach show that artificial injections of milked venom cause an immediate block of spontaneous and evoked motor activity, lasting 30-90 seconds (Haspel *et al*. 2003). In addition, the injection of milked venom into the cockroach terminal abdominal ganglion (A6) blocks cholinergic synaptic transmission and postsynaptic responses to a nicotinic agonist for about 90 seconds (Haspel *et al.* 2003). *Ampulex* venom contains

high concentrations of GABA (25 mM) and its receptor agonists β-alanine (18 mM) and taurine (9 mM), which were determined to be responsible for this transient paralysis by enhancing GABA-mediated chloride ion conductance, resulting in hyperpolarization of central neurons (Moore *et al*. 2006). β-alanine and taurine also enhance the effect of GABA by antagonizing GABA uptake transporters, allowing GABA to remain longer in the synaptic cleft (Hue *et al*. 1981; Whitton *et al*. 1988).

The second sting into the head is responsible for both the compulsive grooming, and long-term hypokinesia. Stung cockroaches groom for approximately thirty minutes and this fixed behavior is caused by the venom. Cockroaches that were grabbed by the wasp and not stung, and cockroaches subjected only to the sting into the prothoracic ganglion groomed significantly less than animals that underwent the full sting repertoire, indicating that compulsive grooming is venom-induced, and not caused by the stress of the attack (Weisel-Eichler *et al.* 1999). The purpose of this behavior is not entirely clear, but this phenomenon may have evolved as a means to prevent the cockroach from moving away before hypokinesia is fully established (Weisel-Eichler *et al.* 1999). This period of time also would allow the wasp to locate a suitable burrow to hide the hypokinesic cockroach away from other predators. Another possible explanation is that grooming cleans the area where oviposition will occur, increasing the chances of successful emergence and survival of the larva.

It has been proposed that a dopamine-like substance in the venom is responsible for the grooming following the head sting. Injections of dopamine and dopamine agonists into the hemolymph and the SEG induce grooming in cockroaches. In addition,

pretreatment with the dopamine receptor antagonist flupenthixol reduces the amount of spontaneous grooming observed after a sting (Weisel-Eichler *et al.* 1999). These results imply that grooming behavior may be the result of dopamine receptor activation. Injection of the plant alkaloid reserpine into the cockroach hemolymph also causes spontaneous grooming. Reserpine acts on the vesicular monoamine transporters (VMAT1 and VMAT2), preventing vesicular uptake of biogenic amines. This causes a massive release, and subsequent depletion, of dopamine, octopamine, and serotonin from presynaptic terminals. Grooming, in response to reserpine injection, is most likely due to a postsynaptic effect of released monoamines (Weisel-Eichler *et al.* 1999). Interestingly, injecting dopamine into a stung animal does not produce excess grooming (Gal *et al*. 2005), suggesting that stung animals are insensitive to dopamine.

The final stage of this behavioral sequence of events is hypokinesia, which is typically characterized by a long-term lethargy and inability of the cockroach to generate wind- or touch-evoked escape responses (Fouad *et al*. 1996). This allows the wasp to manipulate the much larger cockroach, and walk it to its lair with virtually no resistance. When confronted with a noxious stimulus, the threshold to initiate walking was 2-3 fold greater in stung animals than controls (Gal *et al*. 2008). Interestingly, even though the cockroach loses its escape behavior, it retains the ability to right itself when placed on its back, and fly in a wind tunnel (Weisel-Eichler *et al*. 2002). When immersed in a water bath, stung cockroaches initiate swimming much like control animals, but the duration of the activity bout is much shorter (Gal *et al.* 2008). However, recordings from the coxal depressor muscles during swimming bouts show that the motor pattern is unchanged.

This indicates that the venom selectively impairs the neural mechanisms involved in the escape response and the initiation and maintenance of walking, instead of depressing the entire nervous system. This effect is not permanent, and stung animals are able to fully recover in 1-2 weeks, as long as the wasp is removed before oviposition or the egg is removed from the cuticle. The venom also slows metabolism of the cockroach, prolonging its survival and providing more nutrients to the developing larva (Haspel *et al*. 2005).

Venom from *Ampulex compressa* contains a diverse mixture of biological compounds, of which only a few have been identified and characterized. In addition to GABA, β-alanine, and taurine, a dopamine-like substance (Weisel-Eichler *et al.* 1999) (confirmed to be dopamine, see Chapter 4) is present. In addition, four novel peptides with masses of 2807 Da, 2847 Da, 5842 Da, and 7577 Da have been isolated and sequenced, but their functions are currently unknown (Moore 2003). More information regarding venom components and their functions is necessary to understand how the venom interacts with the cockroach central nervous system.

Like the digger wasp, *Ampulex compressa* has evolved to sting directly into the central nervous system of its host. The wasp stinger searches for neuronal tissue in the cockroach head, because removing the brain causes a 15-fold increase in the time spent stinging (Gal *et al.* 2005). The location of venom injection was identified by generating "hot" wasps; that is, wasps with ^{14}C radiolabeled amino acids injected into the abdomen. These wasps were allowed to sting cockroaches, and the radioactivity was visualized in the cockroach head neuronal tissue via autoradiography. The vast majority of

radioactivity in the brain was detected ventral to the central complex, near the mushroom bodies, and the midline of the SEG (Haspel *et al*. 2003). The stinger is about 2.5 mm long, which is sufficiently long to reach the central complex of the brain by going directly through the SEG. This raises the question as to what role the SEG plays in envenomation, and whether the wasp is specifically targeting the tissue or merely piercing it in order to reach the brain.

Locomotor Control in the Central Nervous System

The insect subesophageal ganglion has been implicated in many different behaviors, including flight (Ramirez *et al*. 1988; Gal *et al*. 2006) and walking (Strausfeld 1999; Gal *et al.* 2006). Animals with inactivated SEG-descending interneurons due to a severed SEG (referred to as SEG-less cockroaches) were unable to terminate flight and displayed decreased levels of spontaneous movement and walking. The escape response in SEG-less cockroaches was also compromised (Gal *et al.* 2006). However, cockroaches with brains removed showed no flight behavior when stimulated with gusts of wind, and walked for significantly longer bouts than controls, SEG-less cockroaches and headless cockroaches.

The behaviors of SEG-less cockroaches resemble animals stung by *Ampulex compressa*, in that the escape response and levels of spontaneous locomotion are dramatically reduced. However, SEG ablation does not fully explain venom-induced hypokinesia. Neither brainless, nor SEG-less cockroaches were able to right themselves when placed on their backs, whereas stung cockroaches can. Interestingly, SEG-less

cockroaches often initiated flight rather than the normal righting response (Gal *et al.* 2006), a phenomenon that is not seen in stung animals. The digger wasp, *Liris niger*, also stings into the SEG of its cricket prey, which appears to be responsible for the suppression of spontaneous behavior (Gnatzy 2001), demonstrating that locomotor behavior can be suppressed by an animal venom. It is quite possible that *Ampulex* venom is inducing hypokinesia by altering normal SEG activity and inactivating the descending inputs to the rest of the central nervous system.

The central complex of the brain is also important in the regulation of many behaviors, including courtship behavior (Popov *et al*. 2003), visual learning (Wang *et al*. 2008) and locomotion (Martin *et al*. 1999). It is comprised of four regions: the ellipsoid body, the fan-shaped body, the protocerebral bridge, and the paired noduli (Hanesch *et al*. 1989). Perturbing the protocerebral bridge by expressing tetanus toxin in central complex neurons in *Drosophila melanogaster* resulted in decreased levels of spontaneous locomotion (Martin *et al.* 1999). However, the initiation of activity was unaffected, as the protocerebral bridge defects appeared to affect the maintenance of walking bouts, leading to an overall reduced level of locomotion. *Drosophila* strains with mutations affecting neural architecture of the central complex displayed decreased levels of locomotion (Martin *et al.* 1999; Strauss 2002; Poeck *et al.* 2008). Octopamine has been implicated in many motor behaviors (Saraswati *et al*. 2004; Fussnecker *et al*. 2006), and insects with complex motor behaviors tend to have an intricate system of octopaminergic neuropils in the central complex (Strausfeld 1999). Because *Ampulex* venom is injected

near the central complex, it is conceivable that venom-induced hypokinesia may be caused by disruption of the normal functions of this brain region.

Biogenic Amines and Locomotion

Biogenic amines have important roles as neurotransmitters and neuromodulators in the central nervous system, and have been implicated in many different types of insect behaviors, including locomotion (Yellman *et al*. 1997; Draper *et al*. 2007), grooming (Weisel-Eichler *et al.* 1999), arousal states (Andretic *et al*. 2005; Kume *et al*. 2005), flight (Brembs *et al*. 2007), aggression (Stevenson *et al*. 2005) and reproductive functions (Lee *et al*. 2003). The SEG and the central complex, along with many other regions of the brain, contain aminergic neurons. Serotonin has been detected in the central complex of honeybee (Schurmann *et al*. 1984; Seidel *et al*. 1996), whereas octopamine was detected in the central complex of fruit flies (Monastirioti *et al*. 1995; Sinakevitch *et al*. 2006), cockroaches, and honeybees (Sinakevitch *et al*. 2005). Tyrosine hydroxylase, the rate limiting enzyme for dopamine biosynthesis, has been found in neurons throughout the protocerebrum, with nerve fibers innervating many areas of the brain, including the central complex (Granholm *et al*. 1995). The prevalence of these amines in many areas of the central nervous system underscores their importance in the regulation and maintenance of complex behaviors.

Dopamine, octopamine, and serotonin play important modulatory roles in locomotor processes and in the escape behaviors of insects. In cockroaches, octopamine and dopamine increase synaptic transmission between the ventral giant interneurons and

thoracic interneurons which are involved in the escape response (Casagrand *et al*. 1992). However, serotonin decreases synaptic excitability when applied to the same neuronal circuit. Dopamine also increases thoracic motor neuron response to wind stimulation of the cerci, whereas serotonin decreased motor neuron response (Goldstein *et al*. 1991). In crickets, escape behavior was enhanced by serotonin depletion, whereas dopamine depletion suppressed the escape response (Stevenson *et al*. 2000). All three amines stimulated walking and grooming in decapitated fruit flies, whereas D1- and D2-like dopamine receptor antagonists induced akinesia (Yellman *et al.* 1997). Fruit flies with temperature-sensitive mutations in the pale *(ple)* gene encoding tyrosine hydroxylase showed reduced locomotor activity at the restrictive temperature (Pendleton *et al*. 2002), indicating that inhibition of dopamine formation adversely affects movement and locomotion.

Insect Dopamine Receptors

Biogenic amine receptors are generally rhodopsin-like G-protein coupled receptors. In mammals, five different dopamine receptors have been classified (D1-D5) and grouped into two different families: D1-like receptors and D2-like receptors. Activation of D1-like receptors stimulates adenylate cyclase, leading to increased levels of cAMP, whereas activation of D2-like receptors inhibits adenylate cyclase, decreasing the level of intracellular cAMP. However, there is evidence that dopamine receptors can couple to different Gα proteins (Kimura *et al.* 1995; Sidhu *et al.* 1998; Obadiah *et al.* 1999).

Whereas dopamine receptors have been well studied in mammals, the properties of insect dopamine receptors are just starting to be characterized. Dopamine receptors have been identified and characterized in many different insects, including *Apis mellifera* (Blenau *et al*. 1998; Mustard *et al*. 2003; Beggs *et al*. 2005), *Bombyx mori* (Ohta *et al*. 2009), *Ctenocephalides felis* (Gerber *et al*. 2006), *Manduca sexta* (Granger *et al*. 2000) and *Drosophila melanogaster* (Sugamori *et al*. 1995; Feng *et al*. 1996; Han *et al*. 1996; Hearn *et al*. 2002). These receptors share similar properties to mammalian dopamine receptors, but differences in pharmacological profiles indicate that insect dopamine receptors have distinct qualities.

dDA1 from *Drosophila melanogaster* (also called DmDop1 or DopR) is a 385 amino acid protein that has 49-53% similarity to vertebrate D1-like receptors. dDA1 and DmDop1 are two proteins with identical properties, except that the N-terminus of DmDop1 is extended by 126 amino acids (Gotzes *et al*. 1996). This G-protein coupled receptor is positively coupled to adenylate cyclase, leading to an increase of cAMP upon dopamine receptor binding (Gotzes *et al*. 1994). COS-7 cells expressing this receptor had varying levels of cAMP production in response to agonist stimulation. Interestingly, when expressed in insect Sf9 cells (*Spodoptera frugiperda*) dopamine was able to induce cAMP production with an EC_{50} that was 10-fold less than what was required in mammalian COS-7 cells (Sugamori *et al.* 1995). This showed that Sf9 cells are preferable for pharmacological analysis over mammalian cells, possibly due to Sf9 cells having a cellular environment similar to *Drosophila* neurons. The pharmacological profile of this receptor is indicative of a D1-like receptor, with dopamine being the most

potent ligand (EC_{50} ~ 300 nM in Sf9 cells). Other D1 agonists, including 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene (6,7-ADTN), apomorphine, and L-dihydroxyphenylalanine (L-DOPA), were able to stimulate cAMP production, albeit with varying levels of efficacy (Sugamori *et al.* 1995). One interesting pharmacological property is that this receptor shows poor affinity for the D1-like receptor antagonist SCH-23390, which normally binds to mammalian D1-receptors with high affinity. This further illustrates that insect dopamine receptors are pharmacologically distinct from their vertebrate counterparts.

The honeybee analogue to dDA1, called AmDop1, consists of 402 amino acids and shares 93% homology with the transmembrane regions of dDA1 (Blenau *et al.* 1998). Stimulation of the receptor expressed in human embryonic kidney (HEK 293) cells with dopamine, or the agonist 6,7-ADTN increased the levels of intracellular cAMP, whereas dopamine-induced cAMP elevation was suppressed by flupenthixol. As in dDA1, SCH-23390 showed relatively poor affinity for this receptor, because this antagonist was less efficacious than flupenthixol, butaclamol, and even the D2-selective antagonist spiperone (Blenau *et al.* 1998). Interestingly, cells transfected with AmDop1 had higher levels of basal cAMP than nontransfected cells, indicating that AmDop1 has some level of constitutive activity (Mustard *et al.* 2003).

The location of dDA1 in the central nervous system of *Drosophila melanogaster* was determined by antibody staining. In larvae, staining was seen in the mushroom bodies of the brain, in 4-6 neurons of the subesophageal ganglion, and in each hemisegment of the thoracic and abdominal ganglia (Kim *et al*. 2003). Staining in the

adult central nervous system showed similar results to larval staining, but strong staining was also observed in the central complex. In honeybees, in situ hybridization revealed AmDop1 expression in the mushroom bodies, optic lobes, antennal lobes, and the subesophageal ganglion (Blenau *et al.* 1998). The widespread distribution of this receptor in the brain suggests its importance in higher order functions, and possibly in the regulation of motor processes as well.

The DAMB receptor (also called DopR99B) is a 538 amino acid protein that is specifically found in the mushroom bodies, and is conspicuously absent from the rest of the central nervous system (Han *et al.* 1996). Sequence comparison between DAMB and other G-protein coupled receptors showed significant sequence similarity to vertebrate and invertebrate dopamine receptors, and equal levels of similarity with other biogenic amine receptors (Feng *et al.* 1996). Han *et al*. showed that *Drosophila* S2 cells transfected with this receptor accumulated cAMP. Additionally, it was shown that cAMP elevation was accompanied by an increase in intracellular Ca^{2+} (Feng *et al*. 1996). Endogenous catecholamines were able to activate both signaling pathways, but the pharmacological profile for each pathway was different in response to different synthetic agonists. For example, SKF 82958 was very effective in increasing intracellular calcium, but did not elevate cAMP levels (Reale *et al*. 1997). Although this receptor shares some sequence homology with other *Drosophila* biogenic amine receptors, the pharmacology most closely resembles D1-like dopamine receptors. D1-like receptor agonists and antagonists were generally more effective than D2-like receptor ligands at initiating downstream signaling cascades at this receptor (Feng *et al.* 1996).

AmDop2, the honeybee analogue to DAMB, is also localized to the mushroom bodies of honeybee brains. Much like other insect D1-like receptors, activation of AmDop2 leads to an increase of cAMP. However, dopamine and the synthetic agonist 6,7-ADTN were less potent at AmDop2, than at AmDop1 (Mustard *et al.* 2003). AmDop2 had a higher expression level than AmDop1, and was not constitutively active. The agonists apomorphine, SKF 82958, and 6,7-ADTN were able to increase cAMP levels similar to levels produced by dopamine, but the D2-like agonist lisuride and the vertebrate D1-like agonist SKF 38393 failed to stimulate cAMP production. Flupenthixol was the most effective antagonist tested (Mustard *et al.* 2003).

D2R is currently the only known D2-like dopamine receptor in *Drosophila melanogaster*. Eight different splice variants of this receptor have been identified, varying in size from 461-606 amino acids in length. Dopamine activation of expressed receptors in HEK 293 cells leads to pertussis toxin-sensitive Gi/o-mediated signaling, leading to a decrease in cyclic AMP levels (Hearn *et al.* 2002). Dopamine is the most potent endogenous receptor ligand (EC_{50} = 500 nM), but other biogenic amines were able to stimulate the receptor, albeit with lower potency. Bromocriptine, a D2-selective agonist, activated downstream signaling with full efficacy, whereas many other mammalian agonists, including R(-)-propylnorapomorphine (NPA), 6,7-ADTN, and SKF 82598, only weakly stimulated the receptors. Many mammalian receptor antagonists were tested, but only butaclamol and flupenthixol were able to weakly inhibit the binding of dopamine to the receptor (Hearn *et al.* 2002).

The honeybee analogue to the *Drosophila* D2-like receptor, *Am*DOP3, has similar characteristics to its fruit fly counterpart. Receptor activation leads to a decrease of intracellular cAMP, and bromocriptine was able to mimic the effects of dopamine (Beggs *et al.* 2005). Although the two receptors are quite similar, there are some notable differences between them. *Am*Dop3 has a long extracellular N-terminal end that includes a myristoylation signal. Potential sites for phosphorylation in the third intracellular loop are not conserved between the two receptors, except for the C-terminal end of the loop, which is highly conserved (Beggs *et al.* 2005). Interestingly, basal levels of cAMP are significantly elevated in HEK293 cells transfected with *Am*DOP3, which is unusual because the Gi/o class of G proteins normally downregulates adenylate cyclase.

The locations of D2-like dopamine receptors have been identified in *Drosophila* and *Apis mellifera* by in situ hybridization and antibody staining. AmDop3 from *Apis mellifera* is found in all three regions of the brain, the protocerebrum, deutocerebrum, and tritocerebrum. In the protocerebrum, expression was found in various subsets of Kenyon cells in the mushroom bodies, although a different pattern of expression was seen in adult vs. developing insects (Beggs *et al.* 2005). D2R from *Drosophila* is expressed in the central nervous system in particular cells, including the "Ap-let cohort", which are cells that express the transcription factor *apterous* (Draper *et al.* 2007). D2R is also found in peripheral tissues including the Malpighian tubules and the gut (Draper *et al.* 2007).

Behavioral Aspects of Dopamine Receptor Function

Proper dopamine receptor function is critical for many neurological processes, and many neurodegenerative conditions have been linked to dopamine receptor abnormalities. The dopamine hypothesis of schizophrenia states that the symptoms of schizophrenia are due to overactive dopaminergic signaling, particularly hyperactivity of the D2 receptors (Seeman 1987). Neuroleptic drugs, which target D2 receptors, alleviate psychotic symptoms, whereas the administration of L-DOPA can induce psychotic symptoms (Friedman *et al*. 1991). Positron emission tomography data indicated that the density of D2 receptors in striatal tissue in schizophrenia patients was elevated (Seeman *et al*. 1990), providing solid support for this theory. The D2 receptor density is elevated in Parkinson's disease as well, which may account for clinical hypersensitivity to dopamine receptor agonists. Treatment with L-DOPA reduced the density of D2 receptors to normal levels (Guttman *et al*. 1985), which may be one of the mechanisms by which L-DOPA alleviates Parkinsonian symptoms.

Parkinson's disease is a neurodegenerative disease characterized by the degeneration of dopaminergic neurons in the substantia nigra, leading to a decrease in dopamine production. Clinical symptoms include rigidity, bradykinesia, and the inability to generate spontaneous movements. Animal models aim to replicate the symptoms of the disease by disrupting normal dopaminergic signaling, in order to better understand the etiology of the disease. Drugs like reserpine and methamphetamine lead to dopamine depletion in the nerve terminals, whereas agents like 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) generate oxidative radicals which

selectively kill dopaminergic neurons in the substantia nigra (Betarbet *et al*. 2002). L-DOPA can alleviate the symptoms of Parkinson's disease, because L-DOPA is converted to dopamine in the central nervous system, which can act in place of endogenous dopamine.

D2 receptor deficient mice were originally reported to show Parkinsonian symptoms, including bradykinesia, abnormal posture, and a significant reduction in locomotion (Baik *et al*. 1995). However, another study using independently generated D2-deficient mice did not identify posture abnormalities, bradykinesia, tremors, or ataxia (Kelly *et al*. 1998). These mice did display some locomotor defects, including a decreased ability to initiate spontaneous movements, and overall decreases in traveled distance and time spent in motion. D1 receptor deficient mice are hyperactive (Xu *et al*. 1994) or show no major differences in locomotion compared to wild-type mice (Drago *et al*. 1994). Interestingly, D1 agonist administration to mice overexpressing the D1 receptor led to reduced levels of locomotion and spontaneous movement (Dracheva *et al*. 1999). Knocking down *Drosophila* D2R through RNAi also induced a reduction in locomotor activity which could be rescued by the D2-selective agonist bromocriptine (Draper *et al*. 2007). These data emphasize the importance of dopamine receptors in the maintenance of normal locomotor behavior.

Ampulex Venom-Induced Hypokinesia

Envenomation by the parasitoid wasp *Ampulex compressa* induces behaviors in the cockroach that are phenotypically very similar to Parkinson's disease. Stung

cockroaches show reduced escape responses and diminished ability to generate spontaneous movements. Gal and Libersat proposed that the venom affects locomotion by changing properties of defined sets of neurons in the cockroach central nervous system (Gal *et al.* 2008). The resting potential of octopaminergic neurons was more negative, and the spontaneous firing rate in stung and brainless animals was significantly lower than in control animals (Rosenberg *et al*. 2006). Wind-evoked responses of octopaminergic neurons in the metathoracic ganglion were decreased compared to control animals, and depolarizing pulses, which increase the firing rate of the neurons, induced a firing rate in stung animals that was half the frequency of control animals. Slow coxal depressor (Ds) potentials in the metathoracic leg during swimming had an overall lower discharge rate than those of control animals, which could reduce the amount of movement, because the neurons involved are active during the phase of walking or swimming that produce forward propulsion (Gal *et al.* 2008). Reducing the excitability of octopaminergic neurons may decrease the activity of Ds potentials, which in turn decreases the activity in neurons responsible for walking initiation. Stung animals also had slight, but significant, decreases in the level of fructose 2,6 bisphosphate, indicating that there is less glycolytic activity (Rosenberg *et al.* 2006). This may be responsible for slowing down the cockroach's metabolism and ATP production, which could reduce the amount of available energy needed for escape.

The specific mechanism of action behind hypokinesia induction is currently unknown, but it is very possible that monoaminergic signaling systems are involved. Injections of reserpine into the SEG of the cockroach generate a similar behavior to stung

cockroaches, with a similar time course for recovery (Weisel-Eichler *et al.* 2002). Reserpine injection also causes lethargy and a marked reduction in locomotor activity in crickets (Stevenson *et al.* 2000). The depletion of monoamines interferes with normal aminergic signaling via the removal of endogenous substrates for the monoamine receptors. However, reserpine indiscriminately depletes many different monoamines, many of which may not be involved in hypokinesia induced by the sting. Administration of the dopamine receptor antagonist flupenthixol into the cockroach hemolymph suppressed the escape response, whereas the octopamine receptor antagonist mianserin did not (Weisel-Eichler *et al.* 2002). This indicated that the escape response can be compromised by specifically interfering with dopaminergic signaling. However, injections of the octopamine receptor agonist chlordimeform stimulated walking in stung cockroaches (Rosenberg *et al*. 2007), whereas dopamine receptor agonists had no effect. This indicated that either dopamine had no effect on locomotion in cockroaches, or that stung cockroaches are insensitive to the effects of dopamine. Because dopamine has been implicated in many locomotor processes in insects (Yellman *et al.* 1997; Draper *et al.* 2007; Zhang *et al.* 2007), it is likely that dopamine affects cockroach movement, and that stung cockroaches are simply insensitive to dopamine.

It is quite possible that dopamine insensitivity in stung animals may explain the underlying mechanism behind hypokinesia. Hypokinesic effects can be induced by compromising dopaminergic signaling, either by depleting presynaptic stores of dopamine with drugs, or by treating with dopamine receptor antagonists. A dopamine-like substance has been detected in *Ampulex* venom, so it would not be surprising to see a

change in dopaminergic signaling in response to venom injection. It is unlikely that the venom is killing dopaminergic neurons, because stung animals are able to fully recover in 1-2 weeks.

Envenomation may affect dopaminergic signaling in a number of ways. The venom may disrupt dopamine synthesis or storage, leading to a presynaptic depletion of dopamine. This mechanism would directly lead to Parkinson-like effects, but would not sufficiently explain dopamine insensitivity in stung animals. Another possible mechanism is that the venom is antagonizing postsynaptic dopamine receptors. This would prevent dopamine from initiating a downstream signaling cascade, thus inhibiting any downstream effects mediated by dopamine. Recovery from the effects of hypokinesia may be the result of dopamine receptor turnover. It has been demonstrated that dopamine receptor turnover in rats takes around seven days (Hamblin *et al*. 1983; Leff *et al*. 1984), which is approximately the same time required for recovery from hypokinesia, and the restoration of dopamine-induced grooming in stung animals (Weisel-Eichler *et al.* 2002). Because hypokinesia and dopamine insensitivity have a similar time course of recovery, it is possible that both phenomena stem from the venom's actions upon one molecular target.

The primary aim of this dissertation is to explore the roles of biogenic amines and dopamine receptors in venom-induced hypokinesia in cockroaches stung by jewel wasps. Chapter 2 examines the levels of biogenic amines in stung animals, to determine whether the venom might act in a similar manner as reserpine. Chapter 3 explores the pharmacology of dopamine receptors from *Periplaneta americana* and *Drosophila*

melanogaster, investigating the link between receptor activity and behavioral phenotypes in stung animals. Chapter 4 outlines the search for biological functions and mechanisms of action of *Ampulex* venom. Determining the chemical and biological nature of venom-induced hypokinesia will provide great insight into the biochemical basis of locomotor behavior overall, and perhaps increase our understanding of aminergic signaling.

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Chapter Two

Analysis of Biogenic Amine Levels in *Periplaneta americana* Stung by the Parasitoid Wasp *Ampulex compressa*

Abstract

The emerald jewel wasp, *Ampulex compressa*, uses the American cockroach, *Periplaneta americana*, as a host for its larva. The wasp stings directly into the cockroach brain and subesophageal ganglion to induce a long-term hypokinesia, which reduces the cockroach's ability to escape or generate spontaneous movements. In this state, the wasp walks the cockroach to a burrow, and oviposits predominantly on the coxa of the prothoracic legs with minimal resistance. The mechanism behind hypokinesia induction is not known, but administration of the alkaloid reserpine, which depletes presynaptic stores of monoamines, induces behaviors that resemble venom-induced hypokinesia. Reserpinized cockroaches, like stung animals, have great difficulty generating spontaneous movements, raising the possibility that reserpine and venom may act via the same mechanism of action. High performance liquid chromatography with electrochemical detection (HPLC-ED) revealed that levels of dopamine, serotonin, octopamine and tyramine are significantly depressed in reserpinized animals, but are unchanged in stung animals when compared to controls. This indicates that *Ampulex* venom neither depletes monoamine stores, nor inhibits monoamine synthesis.

Introduction

The solitary wasp *Ampulex compressa* exploits the American cockroach *Periplaneta americana* as a food source for its larvae. In order to subdue the much larger cockroach, the wasp injects venom directly into the central nervous system of the cockroach, inducing a long-term lethargy called hypokinesia. In this hypokinesic state,

the wasp walks the compliant cockroach to a burrow where it carefully lays an egg upon the coxa of the prothoracic legs. The wasp then seals the burrow opening with pebbles and debris. The egg hatches a few days later, and the wasp larva feeds on cockroach hemolymph. After a couple of days, the larva crawls inside the cockroach and feeds on internal tissues until it pupates. Approximately six weeks later, an adult wasp emerges from the hollow shell of the cockroach exoskeleton (Libersat 2003).

Hypokinesia in the stung cockroach is characterized by a reversible long-term lethargy and inability to generate spontaneous movements and respond to external stimuli (Fouad *et al.* 1996). Stung cockroaches do not respond to cercal stimulation, which would normally trigger the escape response and induce running. The threshold for the initiation of walking is elevated, but the motor patterns while walking or swimming are unchanged (Gal *et al.* 2008). Stung animals are able to fully recover in 1-2 weeks if separated from the wasp prior to oviposition (Fouad *et al.* 1994).

Monoamines act as neuromodulators and neurotransmitters in the central nervous system of vertebrates and invertebrates and are intimately connected to locomotor processes. For example, octopamine and dopamine stimulate synaptic transmission in cockroach ventral giant interneurons and thoracic interneurons, which are involved in the escape response, whereas serotonin has the opposite effect (Casagrand *et al.* 1992). Dopamine and serotonin modulate the thoracic motor neuron response to wind stimulation of the cerci (Goldstein *et al.* 1991). Dopamine, serotonin, and octopamine all stimulate walking and grooming in decapitated *Drosophila melanogaster* (Yellman *et al.* 1997), and activation of dopaminergic neurons in fruit flies leads to increased levels of

locomotion (Zhang *et al.* 2007). Tyramine and octopamine regulate flight initiation in flies (Brembs *et al.* 2007), and mutant flies with elevated levels of tyramine and lower levels of octopamine displayed reduced locomotion and movement speed (Saraswati *et al.* 2004). Octopamine and tyramine also modulate honeybee locomotor and grooming behavior (Fussnecker *et al.* 2006).

Hypokinesia induced by *Ampulex* venom phenotypically resembles Parkinson's disease, and impaired dopaminergic signaling is a common feature in animal models of the disease. 6-Hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are neurotoxins used to simulate Parkinson's disease in mammals. These chemicals cause a degeneration of dopaminergic neurons in the substantia nigra, interrupting normal dopaminergic signaling due to decreased levels of dopamine present in the brain (Betarbet *et al.* 2002). Dopamine deficiency in Parkinson's disease leads to bradykinesia and inability to initiate spontaneous movements. Symptoms of Parkinson's disease can be attenuated via the administration of L-3,4-dihydroxyphenylalanine (L-DOPA), which gets converted to dopamine in the body via dopa decarboxylase.

Hypokinesia-like symptoms can be induced by depleting presynaptic stores of biogenic amines. It has been previously demonstrated that injecting reserpine into the cockroach hemolymph causes behavioral effects which are very similar to hypokinesia (Sloley *et al.* 1982; Weisel-Eichler *et al.* 2002). Reserpine antagonizes vesicular monoamine transporters (VMAT1 and VMAT2), thus preventing vesicular loading of dopamine, octopamine, and serotonin in insect nerve terminals (Sloley *et al.* 1982). This

leads to neurotransmitter depletion in the presynaptic terminal, which lasts for weeks (O'Gara *et al.* 1991). Reserpine also depletes presynaptic stores of tyramine (Sasaki *et al.* 2002). High levels of reserpine lead to increased membrane permeability and a release of monoamines into the synaptic cleft (Zallakian *et al.* 1982). Reserpinized cockroaches do not generate spontaneous movements, and respond weakly to touch-evoked stimuli (Weisel-Eichler *et al.* 2002). Similar reserpine-induced behaviors have been observed in crickets (Stevenson *et al.* 2000) and fruit flies (Pendleton *et al.* 2002). Although the mechanism underlying *Ampulex* sting-induced hypokinesia induction in cockroaches is currently unknown, one possibility is that the venom disrupts monoamine storage mechanisms, leading to reduced neuronal levels of monoamines important for locomotion.

Ampulex venom may also inhibit enzymes responsible for biogenic amine synthesis, such as tyrosine hydroxylase or dopa decarboxylase. Inhibiting the biosynthetic pathways of dopamine and serotonin can lead to problems with locomotion. Feeding α-methyl tyrosine, a tyrosine hydroxylase inhibitor, to fruit flies caused a dose dependant decrease in locomotion (Pendleton *et al.* 2005). Fruit flies with mutations in the *pale* gene encoding tyrosine hydroxylase showed locomotor deficits, which could be rescued by administration of L-DOPA (Pendleton *et al.* 2002). Serotonin deficiency in *Caenorhabditis elegans* led to decreased duration of forward movement (Tokumitsu 2005), whereas tyramine deficiency caused defects in reversal behavior (Alkema *et al.* 2005). This indicates that a proper complement of biogenic amines is necessary for proper locomotor behaviors, and disrupting their synthesis can lead to adverse effects.

In this study, we examine levels of dopamine, serotonin, octopamine, and tyramine in stung cockroaches to determine whether altered biogenic amine levels are correlated with venom-induced hypokinesia. High performance liquid chromatography with electrochemical detection (HPLC-ED) is an efficient way to separate and measure amines in biological samples with specificity and sensitivity (Hardie *et al.* 2006). We employed this technique to measure amine levels in the head and thoracic ganglia of cockroaches, and to determine whether envenomation by *Ampulex compressa* could lead to an overall reduction of biogenic amines.

Materials and Methods

Animals. *Ampulex compressa* were maintained in a rearing room at 27° C in 16 in x 16 in x 24 in plexiglass cages with 20-30% humidity. They were fed honey and water separately *ad libitum,* and kept under a 16:8 light-dark cycle. *Periplaneta americana* were raised in garbage cans filled with eggshell cartons on dog food and water *ad libitum*. All experiments were performed on adult male cockroaches.

Treatments. Wasps were allowed to sting individual cockroaches one day before analysis. The wasps performed the entire stinging repertoire in an arena, from which the wasp was removed upon completion of the sting into the head cavity of the cockroach. Reserpine was dissolved in glacial acetic acid at a concentration of 500 mM, and diluted with water to a final concentration of 5 mM (in 1% acetic acid). Ten µL of solution were injected into the abdominal hemocoel, under the third or fourth abdominal sternite. Control cockroaches were kept under the same conditions as stung and reserpinized

animals. As a control for reserpine treatment, sham-injected cockroaches were injected with 10 µL of 1% acetic acid.

Behavior Analysis. To determine the levels of spontaneous movement, cockroaches were placed individually into a circular arena (60 cm in diameter) covered with a transparent sheet of plexiglass to prevent their escape. The cockroach was placed in the center of the arena and allowed to acclimate to its new surroundings for five minutes. Cockroaches were subsequently filmed for a 10 minute period. To determine amounts of spontaneous locomotion, the arena was divided into four quadrants of equal size, and the number of times a cockroach crossed into a new quadrant was recorded. Stung, reserpinized and sham-injected cockroaches were analyzed in the arena one day after their respective treatments.

HPLC Sample Preparation. Extracts of individual thoracic ganglia (T1, T2 and T3 ganglia) and head ganglia (brain and subesophageal ganglion) were prepared from freshly dissected cockroaches by homogenization in 100 µL of 0.1 M perchloric acid. Samples were centrifuged two times at 13000 rpm for 20 minutes, using different tubes each time. Centrifuging two times in different tubes helped remove tissue fragments that did not pellet well, and led to cleaner analyses. Supernatant was collected and kept in the dark on ice until analyzed by HPLC. Samples were analyzed shortly after preparation, with no sample being stored longer than 90 minutes. Dopamine, serotonin, tyramine, and octopamine standards were prepared fresh daily in 0.1 M perchloric acid and diluted in mobile phase.

HPLC Analysis. Extracts (20 µL) were injected by hand onto an ESA model 580 (ESA, Chelmsford, MA) isocratic solvent delivery module connected to a reversed-phase HPLC column and an ESA Coulochem II electrochemical detector. Fractionation was performed using a 250 x 4.6 mm, 5 µm particle size, 120 Å pore size Clipeus C18 reversed-phase column (Higgins Analytical, Mountain View, CA) with a flow rate of 1.0 ml/min. The mobile phase consisted of 50 mM citrate with 10 mM heptanesulfonic acid, 15% acetonitrile adjusted to a pH of 4.5 using sodium acetate (Hardie *et al.* 2006).

To analyze dopamine and serotonin, a series of two porous graphite electrodes, a screening electrode and an oxidizing electrode, were set to potentials of -150 mV and +250 mV respectively. The potential of +250 was strong enough to fully oxidize dopamine and serotonin while leaving many other unidentified electroactive compounds unoxidized, freeing the chromatogram of unwanted clutter. For octopamine and tyramine, the two electrodes were set to potentials of +250 mV and +650 mV respectively. A potential of +650 mV was powerful enough to oxidize tyramine and octopamine. At +250 mV, the screening electrode effectively removed compounds that oxidize at low potentials (such as dopamine), thus clearing the chromatogram of undesired peaks. Data were collected and chromatogram peaks were integrated using System Gold chromatography software (Beckman). Standard curves for known quantities of dopamine, serotonin, octopamine, and tyramine (5, 1, and 0.5 pmoles) were generated daily to ensure accurate estimates of amine levels. Data were quantified by integration of peak areas, and amine levels in biological samples were determined by interpolation of standard curves. As only 20% of total extract was analyzed each run (20

µL of the 100 µL homogenate), we were able to calculate amine content of the entire ganglion. All drugs and reagents were purchased from Sigma (St. Louis, MO).

Statistics. Statistical analyses were performed on all data sets, where parametric data were analyzed with ANOVA and nonparametric data were assessed with the Kruskal-Wallis test with various *post hoc* tests. Because of the low number of replicates, series of two-sample T-tests (for parametric data) or Mann-Whitney Rank Sum tests (nonparametric data) were performed to statistically compare amine levels in stung cockroaches injected with reserpine to levels in stung cockroaches without reserpine treatment and unstung cockroaches treated with reserpine.

Results

Spontaneous Locomotion. Levels of spontaneous activity were determined by placing cockroaches into an arena and recording their movements. Stung animals and animals injected with reserpine did not move at all from their original starting positions. Reserpinized cockroaches occasionally rotated on the axis where they were standing, but did not take any steps forward. Conversely, control animals and sham-injected animals displayed significantly higher levels of activity ($p \leq 0.001$), averaging 11.2 and 15.5 quadrant crossings in ten minutes (Figure 2-1). There were no significant differences in movement between naïve cockroaches, and sham-injected cockroaches.

Chromatographic Conditions. Dopamine and serotonin were analyzed separately from octopamine and tyramine, due to the differences in the potential required for oxidation. The optimal potentials were experimentally determined. An oxidizing potential of 250

mV was sufficient to oxidize dopamine and serotonin, while leaving most other compounds unoxidized and undetected. This allowed dopamine and serotonin to be unambiguously resolved on the chromatograms, without interference from octopamine, tyramine, or tryptophan.

Octopamine and tyramine were oxidized at 650 mV. The screening electrode was set to 250 mV, which efficiently eliminated dopamine from the octopamine/tyramine chromatogram. The screening electrode also removed a compound present in the tissue homogenate that co-eluted with octopamine (under conditions when the screening electrode was not used), ensuring that the corresponding peak on the chromatogram was truly representative of octopamine and nothing else. Compound identity was primarily determined by retention time compared to known standards, and confirmed by current/voltage plots for each amine (data not shown).

Amine Levels in Head Ganglia. Amine levels were measured in both brain and subesophageal ganglia. Control brains contained approximately 18.1 ± 1.6, 13.2 ± 1.0, 19.9 ± 7.0 and 3.4 ± 0.96 pmoles of dopamine, serotonin, octopamine, and tyramine respectively, whereas control SEGs contained 1.6 ± 0.34, 4.3 ± 0.38, 5.8 ± 1.1 and 1.0 ± 0.29 pmoles respectively (Figure 2-3). Amine levels in stung animals do not differ significantly from control levels in either of the head ganglia for any of the amines measured. Animals treated with reserpine have significantly lower levels of all amines in both head ganglia compared to control and stung animals ($p \leq 0.01$). In many cases, the levels of dopamine, serotonin and octopamine were not detectable. Tyramine levels in reserpinized animals were at about 50% of control values in either tissue, although the

differences were significantly different ($p \leq 0.01$). There was no significant difference to controls in sham-injected animals.

Amine Levels in Thoracic Ganglia. The levels of amines varied in the three thoracic ganglia. T1 ganglia from control animals contained 1.32 ± 0.49, 0.9 ± 0.34, 5.38 ± 1.02 and 1.48 ± 0.36 pmoles of dopamine, serotonin, octopamine, and tyramine respectively. The T2 ganglia contained 1.66 ± 0.72, 2.27 ± 0.89, 3.80 ± 1.11, and 2.00 ± 0.54 pmoles of each respective amine, while the T3 ganglia contained 1.18 ± 0.33, 2.54 ± .50, 4.45 ± 1.31, and 1.58 ± 0.39 pmoles of each compound (Figure 2-4). Compared to controls, amine levels were not significantly different in stung animals in any of the ganglia. As in the head ganglia, reserpine significantly reduced amine levels in all three ganglia.

Reserpine Depletes Amines in Stung Cockroach Tissues. Reserpine was injected into cockroaches stung by *Ampulex compressa* to determine whether envenomation compromises aminergic release mechanisms in presynaptic terminals. There was a significant depletion of dopamine and serotonin in the brain and SEG of these animals when compared to amine levels in stung cockroaches that had not been give reserpine (Figure 2-5). There was no significant difference between amine levels in reserpinized animals, and reserpinized animals that had been stung one day earlier.

Discussion

Cockroaches stung by *Ampulex compressa* undergo major behavioral changes, the most prominent of which is the long-term hypokinesia. Hypokinesia is not an overall suppression of motor function, but a selective depression of certain behaviors (Weisel-

Eichler *et al.* 2002). The threshold for initiation of walking in stung cockroaches is elevated, but motor patterns are not altered (Gal *et al.* 2008), indicating that stung cockroaches can walk when led by the wasp, but cannot initiate movement on their own to escape.

Reserpine has been used previously to effectively deplete biogenic amines in animals (Karoum *et al.* 1979; O'Gara *et al.* 1991), and is a useful compound for studying the behavioral effects of amine depletion *in vivo*. Reserpinized cockroaches are a well-characterized behavioral model for venom-induced hypokinesia (Weisel-Eichler *et al.* 2002). Injections of reserpine into the hemolymph or SEG produce animals that fail to generate spontaneous movements, and respond weakly to cercal stimuli. Reserpinized animals also have the same posture as stung animals, and regain proper locomotor function in approximately 2-4 weeks, which is similar in duration to recovery from venom-induced hypokinesia (Weisel-Eichler *et al.* 2002). This underscores the importance of monoaminergic signaling for locomotion and escape, because the disruption of proper monoamine storage leads to long-term behavioral deficits.

In this study, we show that amine levels in stung animals were not statistically different than levels in control animals. This precludes the possibility that *Ampulex* venom depletes presynaptic stores of monoamines, or interferes with monoamine synthesis, because either would lead to decreased levels in the tissue. This result was not completely unexpected, because amine depletion does not explain all the behavioral phenotypes seen in stung animals. Stung animals retain the ability to fly (Fouad *et al.* 1994), whereas reserpinized animals cannot, although this may be due to reserpine's

effects in the entire animal, as opposed to localized effects of the venom in the central nervous system (Weisel-Eichler *et al.* 2002). Stung animals also do not groom in response to dopamine injection, whereas naïve animals groom significantly longer than sham-injected animals (Weisel-Eichler *et al.* 1999; Weisel-Eichler *et al.* 2002).

If sting-induced hypokinesia were elicited by decreasing amine levels in the central nervous system, one would expect to be able to restore locomotion by supplementing stung animals with amines. Even though octopamine levels are unchanged in the head and thoracic ganglia, it has been shown that injections of the octopamine receptor agonist chlordimeform into the brain or hemolymph can stimulate walking in stung animals (Rosenberg *et al.* 2007). Octopamine is known to stimulate many active processes in insects, including locomotion (Yellman *et al.* 1997; Saraswati *et al.* 2004), aggression (Stevenson *et al.* 2005; Hoyer *et al.* 2008), and flight (Kinnamon *et al.* 1984; Brembs *et al.* 2007; Vierk *et al.* 2009), so it is conceivable that octopamine-induced walking in stung animals is not necessarily reversing venom-induced hypokinesia, but merely activating circuitry unrelated to it.

Octopaminergic dorsal unpaired median (DUM) neurons are less excitable in stung animals (Rosenberg *et al.* 2006), which may reduce the amount of octopamine secreted, thus decreasing octopaminergic postsynaptic effects. Injections of chlordimeform would supplement endogenous octopamine, providing more ligands for synaptic activity and subsequently increasing locomotor output. Nonetheless, reduction in DUM neuron excitability is the result of venom injection into the head ganglia,

suggesting that *Ampulex* venom acts on another biological target to inhibit descending inputs from the brain and/or SEG (Rosenberg *et al*. 2007).

Dopamine is known to affect locomotor processes in insects (Yellman *et al.* 1997; Pendleton *et al.* 2002; Zhang *et al.* 2007), and enhances synaptic transmission between ventral giant interneurons and thoracic interneurons involved in the escape response (Casagrand *et al.* 1992). However, injection of dopamine or dopamine agonists failed to stimulate walking in stung animals (Rosenberg *et al.* 2007). The absence of any noticeable effect from dopamine injection into stung animals implies that envenomation renders animals insensitive to the effects of dopamine. Stung cockroaches also fail to engage in dopamine-induced grooming (Weisel-Eichler *et al*. 1999), providing further evidence for dopamine insensitivity.

Ampulex venom acts on the central nervous system to selectively depress certain locomotor behaviors. It is unlikely that venom is preventing the release of biogenic amines from presynaptic terminals, because reserpine treatment effectively reduces dopamine and serotonin levels in stung cockroaches. This suggests that amines remain susceptible to reserpine-induced depletion. In addition, this mechanism would not explain why dopamine injection does not elicit grooming in stung animals. It is more likely that *Ampulex* venom is somehow antagonizing postsynaptic dopamine receptors. This would explain dopamine insensitivity in stung animals, and also disrupt dopaminergic signaling, leading to Parkinson-like symptoms. Dopamine receptor turnover in rats takes around 7-10 days (Hamblin *et al.* 1983). It has been shown that recovery from venom injection can range from 1-4 weeks (Fouad *et al.* 1994), but we

have observed animals recover in as little as one week, the same time course for receptor turnover in rats. Treating cockroaches with dopamine receptor antagonists suppressed the escape response (Weisel-Eichler *et al.* 2002), showing that dopamine receptor dysfunction can be responsible for the major symptoms of hypokinesia. The role of dopamine receptors in the behavioral modification caused by envenomation is the subject of the next chapter.

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Figure 2-1. Effects of reserpine and venom on spontaneous locomotion. Stung and reserpinized cockroaches displayed no spontaneous movement over a period of ten minutes. There were no significant differences between control animals and animals injected with 1% acetic acid, the carrier for reserpine. *** indicates a significant difference ($p \leq 0.001$, n=10) using the Kruskal-Wallis analysis followed by *post hoc* Tukey's test. Error bars represent standard deviations from the mean.

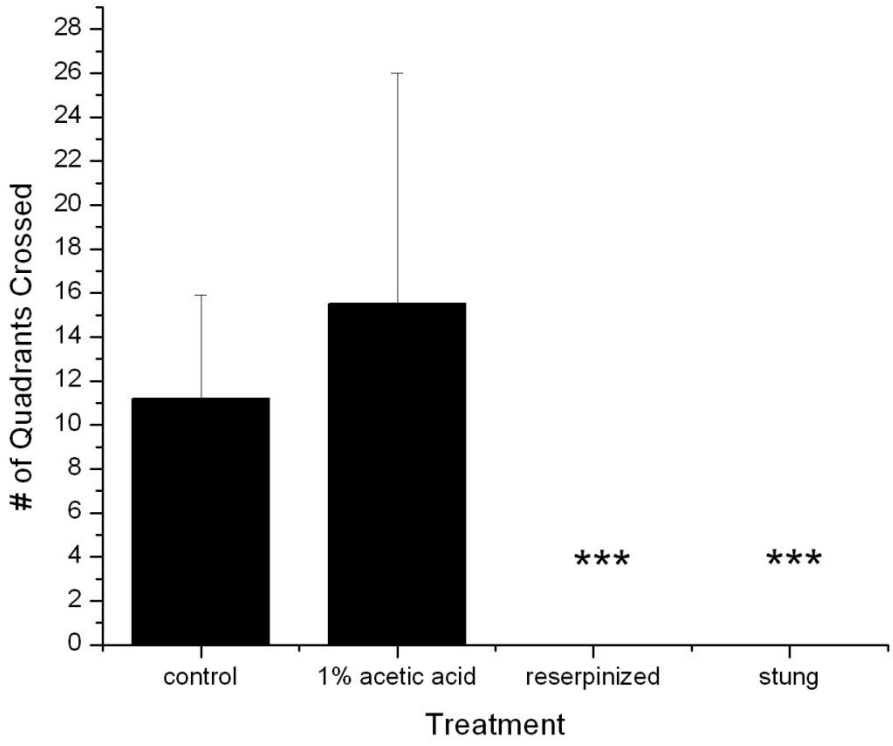
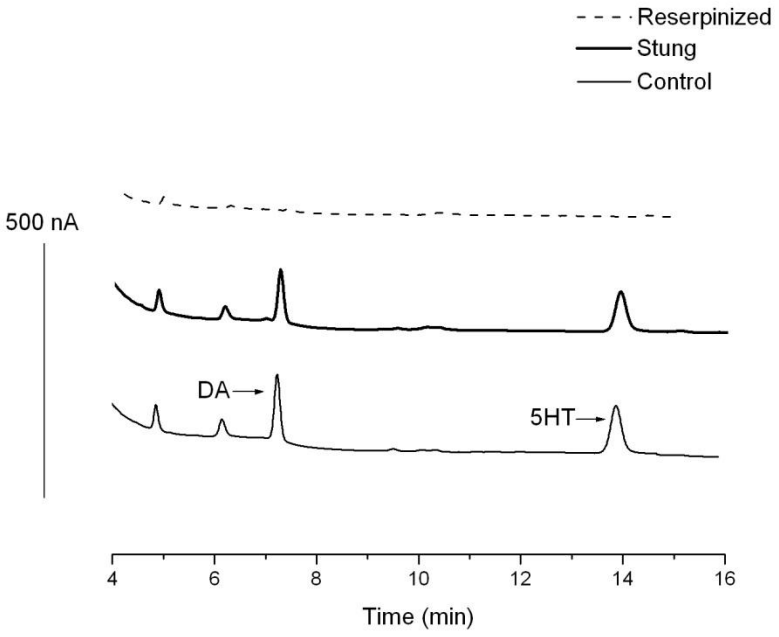

# of Quadrants Crossed
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
***
***
control
1% acetic acid
reserpinized
stung
Treatment

Figure 2-1

Figure 2-2. **A,** Sample chromatograms from cockroach brain showing peaks for dopamine (DA) and serotonin (5HT). There are no significant differences between dopamine and serotonin levels in stung and control animals. In reserpinized animals, the dopamine and serotonin peaks are significantly reduced. **B,** Sample chromatograms from cockroach brain showing octopamine (OA) and tyramine (TA) peaks. There are no significant differences in octopamine and tyramine levels between stung and control animals. In reserpinized animals, octopamine is nearly fully depleted, while tyramine levels are significantly lower, but still present at detectable levels.

A


Reserpinized
Stung
Control
500 nA
DA
5HT
4
6
8
10
12
14
16
Time (min)

B

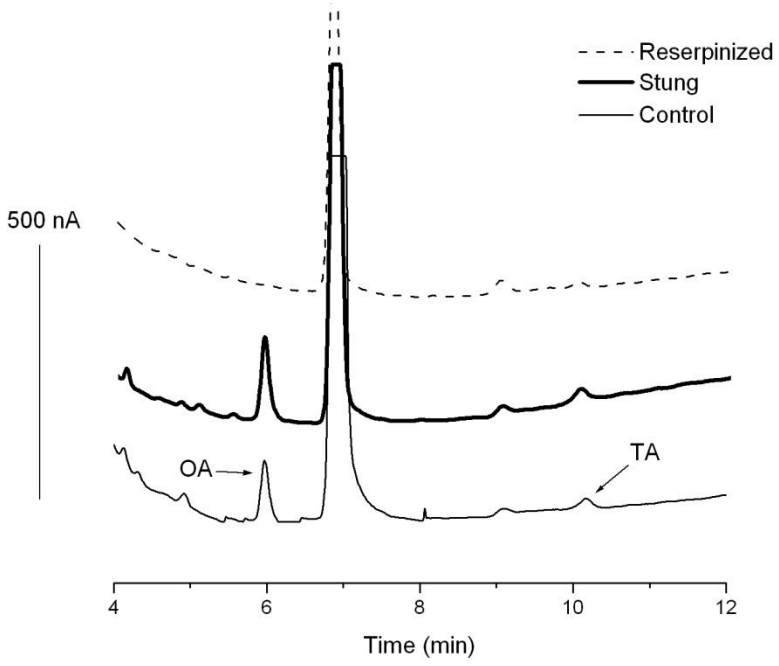
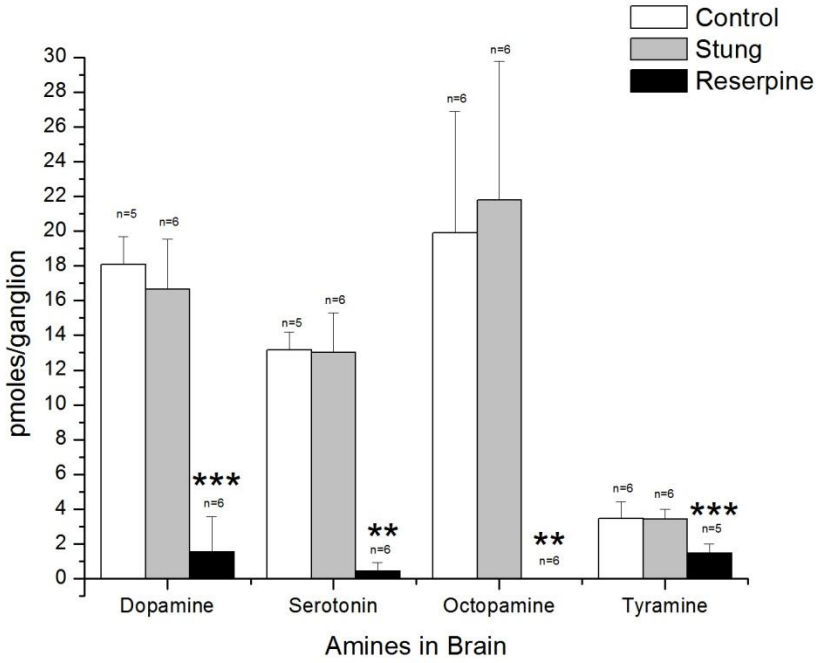

Reserpinized
Stung
Control
500 nA
OA
TA
4
6
8
10
12
Time (min)

Figure 2-2

Figure 2-3. Head ganglia measurements in control, stung, and reserpinized animals. **A**, Levels of dopamine, serotonin, octopamine, and tyramine in the brain. There were no significant differences between control and stung animals, but reserpine significantly reduced levels of all amines examined. Octopamine was not detected in any reserpinized brain samples. **B**, Amine levels in the subesophageal ganglion (SEG). Amine levels were not significantly different in control and stung animals, but were significantly depressed in reserpinized animals. A one-way ANOVA with a *post hoc* Holm-Sidak test was performed for the following: dopamine in the brain, serotonin in the SEG, and tyramine in the brain. A Kruskal-Wallis analysis with a *post hoc* Tukey's test was performed for the following: dopamine in the SEG, and octopamine in the brain and SEG. A Kruskal-Wallis analysis with a *post hoc* Tukey's test was performed to measure serotonin in the brain. ** indicates $p \leq 0.01$, while *** indicates $p \leq 0.001$. Error bars represent standard deviations from the mean.

A


Control
Stung
Reserpine
pmoles/ganglion
Dopamine
Serotonin
Octopamine
Tyramine
Amines in Brain

B

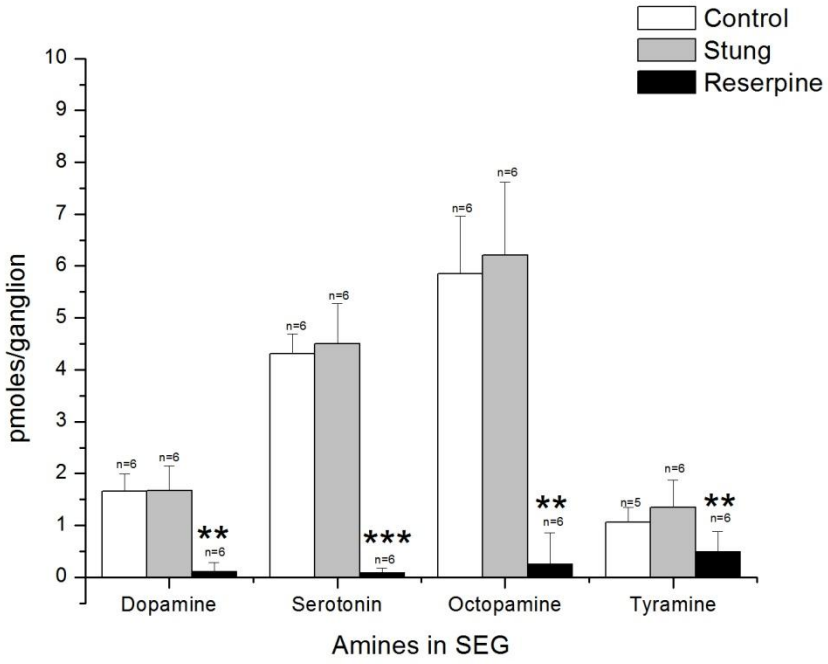

Control
Stung
Reserpine
pmoles/ganglion
Dopamine
Serotonin
Octopamine
Tyramine
Amines in SEG

Figure 2-3

Figure 2-4. Amine levels in the prothoracic (T1) ganglion. There were no significant differences in amine levels between control and stung animals, but reserpinized animals had significantly lower levels of each amine. In reserpinized animals, serotonin and tyramine levels were not detected, whereas dopamine levels were detectable in only one animal. A one-way ANOVA with a *post hoc* Holm-Sidak test was performed to statistically assess octopamine levels. A Kruskal-Wallis analysis with a *post hoc* Tukey's test was performed to statistically assess dopamine and serotonin levels. A Kruskal-Wallis analysis with a *post hoc* Dunn's test was performed to statistically assess tyramine levels. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$. Error bars represent standard deviations from the mean.

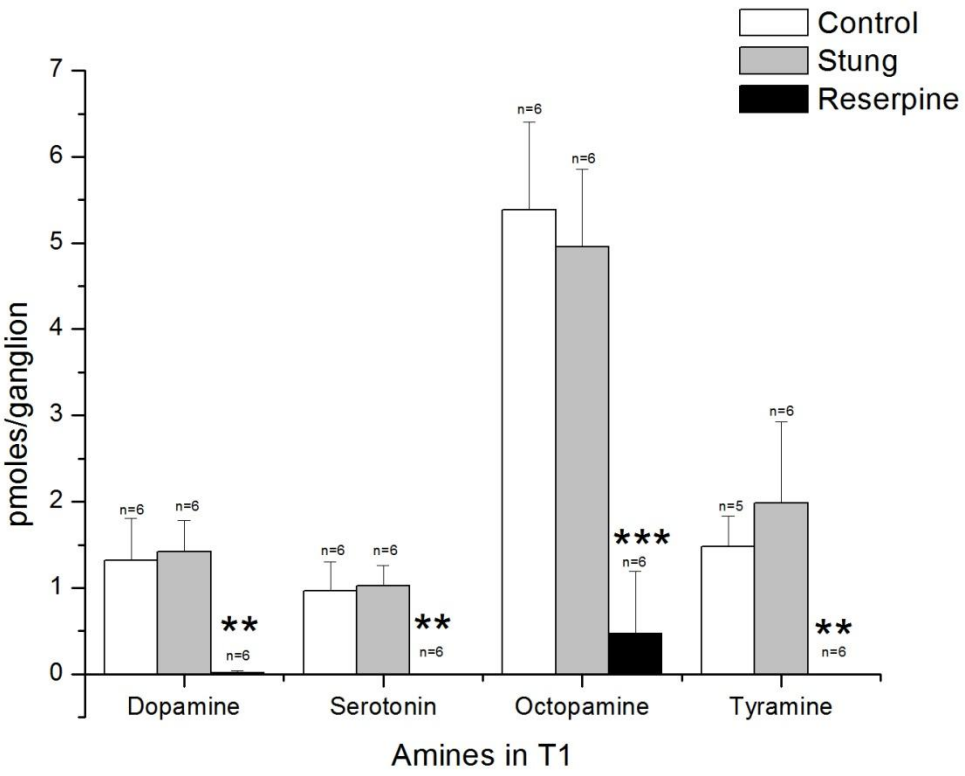

Control
Stung
Reserpine
pmoles/ganglion
0
1
2
3
4
5
6
7
n=6
n=6
**
n=6
n=6
n=6
**
n=6
n=6
n=6
***
n=6
n=5
n=6
**
n=6
Dopamine
Serotonin
Octopamine
Tyramine
Amines in T1

Figure 2-4

Figure 2-5. Amine levels in the mesothoracic (T2) ganglion of control, stung, and reserpinized cockroaches. There were no significant differences between stung and control animals, but reserpinized animals had significantly lower levels of each amine. Dopamine and serotonin were not detected in reserpinized animals. A one-way ANOVA with a *post hoc* Holm-Sidak test was performed to statistically assess tyramine levels. A Kruskal-Wallis analysis with a *post hoc* Tukey's test was performed to statistically assess dopamine levels. A Kruskal-Wallis analysis with a *post hoc* Dunn's test was performed to statistically assess octopamine levels. * indicates $p \leq 0.05$. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$. Error bars represent standard deviations from the mean.

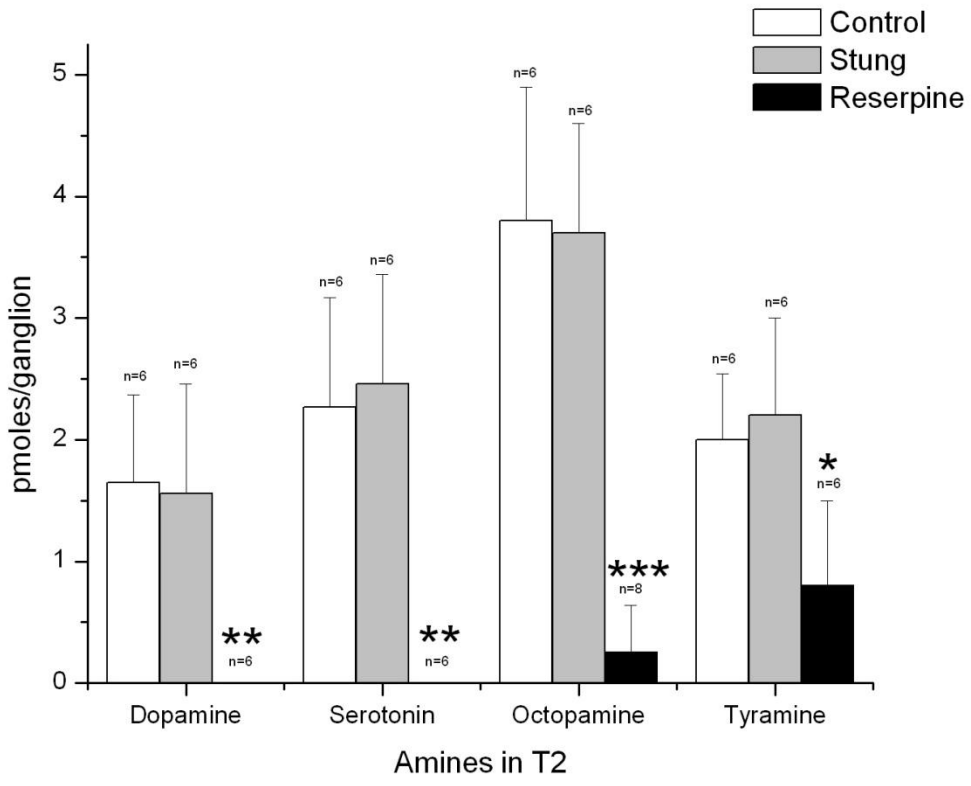

Control
Stung
Reserpine
pmoles/ganglion
0
1
2
3
4
5
n=6
n=6
**
n=6
n=6
n=6
**
n=6
n=6
n=6
***
n=8
n=6
n=6
*
n=6
Dopamine
Serotonin
Octopamine
Tyramine
Amines in T2

Figure 2-5

Figure 2-6. Amine levels in the metathoracic (T3) ganglion in control, stung, and reserpinized animals. There were no significant differences between amine levels in control and stung animals, but reserpinized animals had significantly lower levels of each amine. A one-way ANOVA with a *post hoc* Holm-Sidak test was performed to statistically assess dopamine and tyramine levels. A Kruskal-Wallis analysis with a *post hoc* Tukey's test was performed to statistically assess serotonin and octopamine levels. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$. Error bars represent standard deviations from the mean.

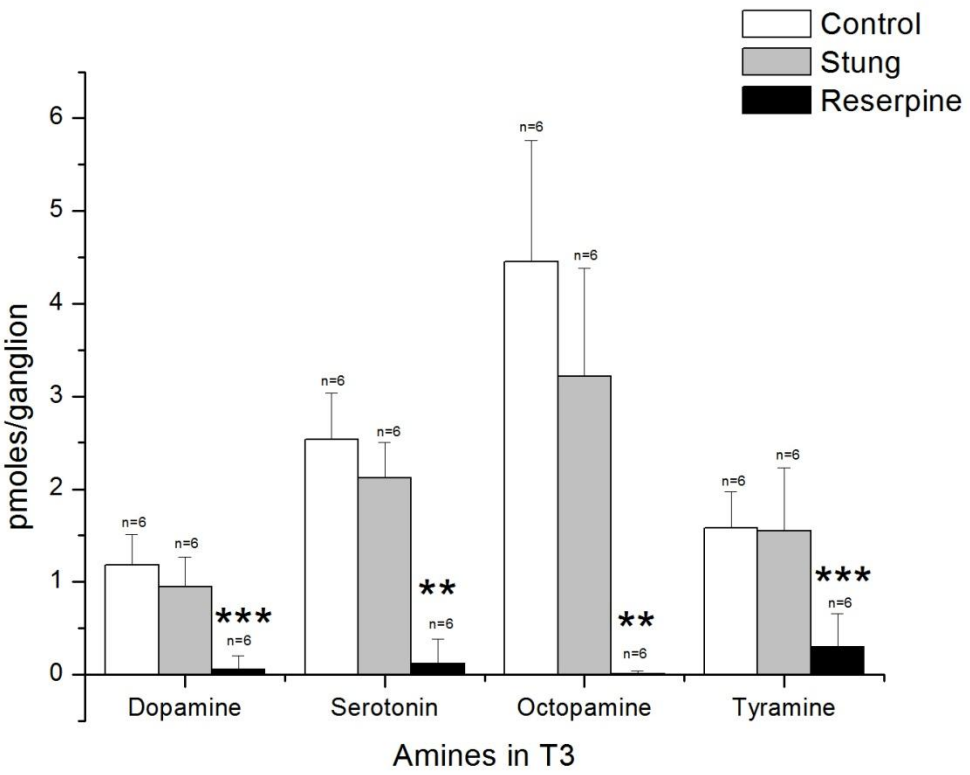
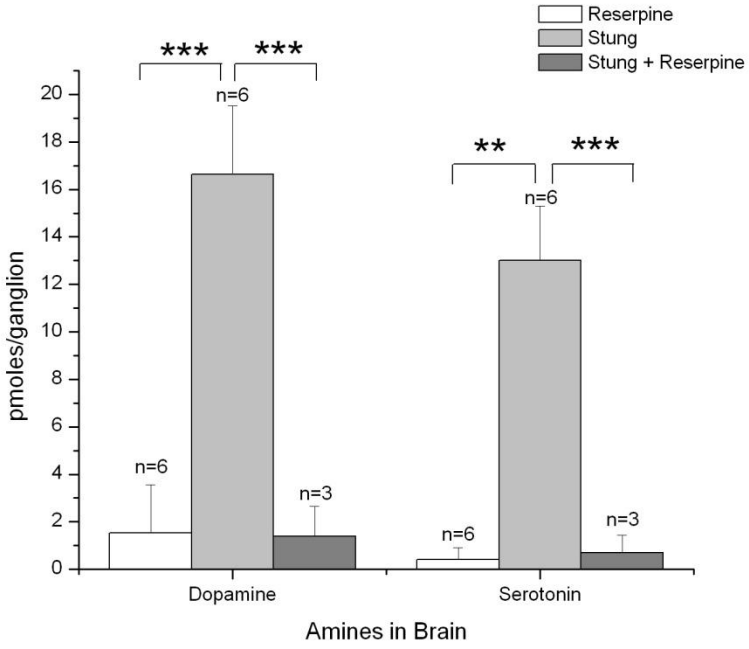

Control
Stung
Reserpine
pmoles/ganglion
0
1
2
3
4
5
6
n=6
***
**
**
***
Dopamine
Serotonin
Octopamine
Tyramine
Amines in T3

Figure 2-6

Figure 2-7. Amine measurements in **A**, brain and **B**, subesophageal ganglion of stung cockroaches injected with reserpine, compared to stung cockroaches (with no reserpine) and naive cockroaches injected with reserpine. Reserpine acts independently of the sting, significantly depleting dopamine and serotonin levels. There were no significant differences between stung cockroaches injected with reserpine, and unstung cockroaches injected with reserpine. Because the sample size of stung animals injected with reserpine was low (n=3), two sample T-tests (for parametric data) and Mann Whitney Rank Sum tests (for nonparametric data) were performed to determine statistical significance. * indicates $p \leq 0.05$. ** indicates $p \leq 0.01$. *** indicates $p \leq 0.001$. Error bars represent standard deviations from the mean.

A


Reserpine
Stung
Stung + Reserpine
***
***
**
***
n=6
n=6
n=3
n=6
n=6
n=3
pmoles/ganglion
0
2
4
6
8
10
12
14
16
18
20
Dopamine
Serotonin
Amines in Brain

B

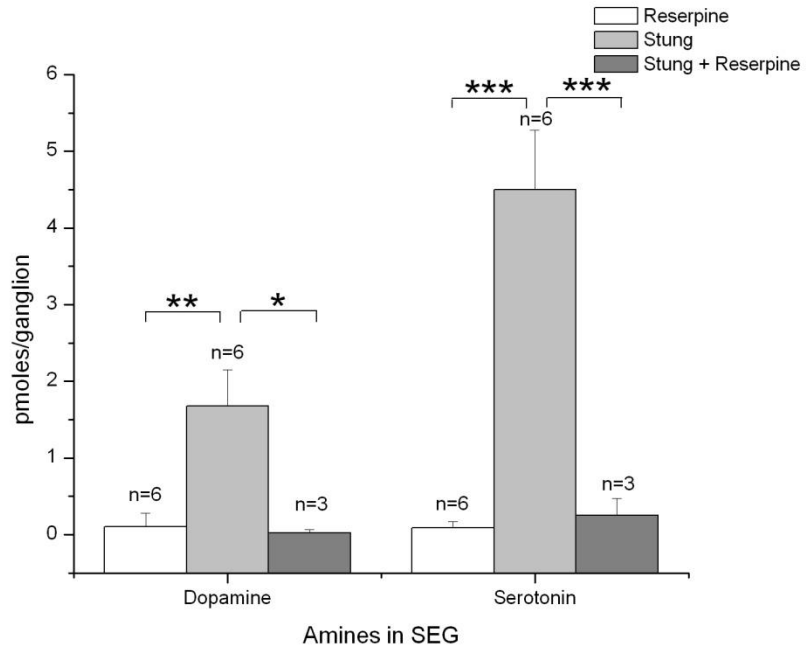

Reserpine
Stung
Stung + Reserpine
***
***
**
*
n=6
n=6
n=3
n=6
n=6
n=3
pmoles/ganglion
0
1
2
3
4
5
6
Dopamine
Serotonin
Amines in SEG

Figure 2-7

Chapter Three

Molecular and Pharmacological Analysis of a D2-Like Dopamine Receptor from the American Cockroach *Periplaneta americana* and a Comparative Study of Three Dopamine Receptors from *Drosophila melanogaster*

Abstract

Dopamine is an important neurotransmitter in the central nervous system that is involved in many different physiological and behavioral processes, including locomotion, grooming, and reproductive functions. Much like in mammals, dopaminergic signaling in insects is mediated through dopamine receptors, which are rhodopsin-like G-protein coupled receptors. The parasitoid wasp *Ampulex compressa* modifies the locomotor behavior of the American cockroach, *Periplaneta americana*, by injecting venom directly into the brain and subesophageal ganglion of the host. Stung cockroaches compulsively groom directly after the sting, and subsequently fall into a state of hypokinesia, which is characterized by a dramatic reduction in escape response and ability to generate spontaneous movements. Circumstantial evidence implicates the dopaminergic system with venom-induced grooming and hypokinesia. In order to associate dopamine receptors with the venom-induced hypokinesic state, I sought to characterize dopamine receptors in the cockroach CNS. The D2-like dopamine receptor from *Periplaneta americana* and three known dopamine receptors from *Drosophila melanogaster* (dDA1, DAMB, and D2R) were cloned and expressed *in vitro* to establish their respective pharmacological profiles using an aequorin-based luminescence assay, which allowed real time measurements of receptor activation. Dopamine was the most potent ligand at each receptor, but the D2-like receptor also was activated by other biogenic amines, such as norepinephrine and serotonin. The agonist bromocriptine, and the antagonists sulpiride and fallypride, were selective for insect D2-like receptors, whereas a number of mammalian D1- and D2-like selective agonists displayed nonselective activity in insect

systems, illustrating that insect dopamine receptors exhibit profiles distinct from their mammalian counterparts. Pretreating cockroaches with the D2-selective antagonist sulpiride slightly, but significantly, reduced the duration of *Ampulex* venom-induced grooming, whereas the D2-selective agonist bromocriptine, and the nonselective antagonists SCH 23390 and flupenthixol, had no observable effect.

Introduction

Dopamine is an essential neurotransmitter and neuromodulator in the central nervous system of vertebrates and invertebrates. Dopamine function has been linked to many physiological and behavioral processes, including locomotion (Yellman *et al.* 1997; Draper *et al.* 2007), grooming (Weisel-Eichler *et al.* 1999; Berridge *et al.* 2000a; Berridge *et al.* 2000b), reproductive functions (Melis *et al.* 1995; Neckameyer 1998), arousal states (Kume *et al.* 2005), and learning (Neckameyer 1998).

Dopamine exerts its functions through interaction with dopamine receptors, which are rhodopsin-like G-protein coupled receptors (GPCRs). In mammals, five subclasses of dopamine receptors (D1 – D5) have been identified and grouped into two families: D1-like receptors (D1, D5) and D2-like receptors (D2, D3, D4). This classification is based on amino acid sequence similarities of transmembrane domains, pharmacological profiles, and downstream signaling pathways (Civelli *et al.* 1993; Gingrich *et al.* 1993; Missale *et al.* 1998). D1-like receptors are linked to Gαs proteins, which stimulate adenylate cyclase, causing an increased production of cAMP from ATP. D2-like receptors are linked to Gαi/o proteins, which inhibit adenylate cyclase from making

cAMP (Missale *et al.* 1998). It has been shown, however, that some dopamine receptors can couple to multiple Gα proteins, leading to different downstream signaling cascades (Kimura *et al.* 1995; Sidhu *et al.* 1998; Obadiah *et al.* 1999).

Dopaminergic signaling mediates many important biological processes, and many different neurological problems are associated with dopamine receptor dysfunction. Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons in the substantia nigra. Reduced levels of dopamine in the basal ganglia are responsible for the typical symptoms of PD, which include bradykinesia, rigidity, and the inability to generate spontaneous movements (Bernheimer *et al.* 1973; Hastings *et al.* 1997). Elevation of postsynaptic D2 receptor density is also a common feature of the disease (Seeman *et al.* 1990). Treatment with L-DOPA, which is converted into dopamine in the central nervous system, reduced D2 receptor density to normal levels, and was efficient in treating symptoms of PD (Guttman *et al.* 1985).

D2 receptor knockout mice were initially reported to display Parkinsonian symptoms (Baik *et al.* 1995), but a subsequent study using independently generated D2-deficient mice did not identify posture abnormalities, tremors, bradykinesia, or ataxia (Kelly *et al.* 1998). Both studies reported locomotor defects and decreased ability to initiate movement in receptor-deficient mice, although the latter reported behavioral deficits that were less severe than in animals treated with haloperidol, a D2 receptor antagonist. Knocking down the D2-like receptor in *Drosophila melanogaster* via RNA interference (RNAi) reduced levels of locomotion which could be rescued by the D2-selective agonist bromocriptine (Draper *et al.* 2007). D1 receptor knockout mice were

mildly hyperactive (Xu *et al.* 1994) or displayed no observable differences in locomotion (Drago *et al.* 1994). However, administration of D1-selective antagonists led to overall reductions in mobility and locomotion (Meyer *et al.* 1992; Meyer *et al.* 1993), demonstrating that D1 receptors also are important for proper locomotion.

The parasitoid wasp *Ampulex compressa* uses its venom to modify the behavior of the American cockroach, *Periplaneta americana*. Stung cockroaches become hypokinesic, and lose their escape response and ability to generate spontaneous movements. However, stung animals retain other behaviors, such as the ability to fly in a wind tunnel and the righting response (Weisel-Eichler *et al.* 2002). This indicates that a select subset of behaviors is affected by the venom, and hypokinesia is not simply a global depression of motor activity. Stung cockroaches can be induced to walk with applications of noxious stimuli, but the threshold for walking initiation is greatly elevated (Gal *et al.* 2008). However, the motor pattern is unchanged, indicating that stung cockroaches retain the physical ability to walk with their normal gait, but simply lack the motivation to initiate movement.

Hypokinesia induced by *Ampulex* venom resembles the symptoms of neurodegeneration brought on by Parkinson's disease. Injections of the plant alkaloid reserpine generated Parkinsonian symptoms in both mammals and cockroaches by depleting synaptic stores of biogenic amines (Betarbet *et al.* 2002; Weisel-Eichler *et al.* 2002). However, venom did not deplete stores of amines, indicating that the venom must have a different mechanism of action (Chapter 2). It is possible that venom disrupts normal dopaminergic signaling through the antagonism of postsynaptic dopamine

receptors, which has been demonstrated to induce hypokinesic symptoms in cockroaches (Weisel-Eichler *et al.* 2002).

Dopamine receptors have been implicated in certain behaviors that are affected by the wasp sting into the brain. Injection of the nonselective dopamine receptor antagonist flupenthixol into cockroach hemolymph suppressed the escape response (Weisel-Eichler *et al.* 2002). Injections of dopamine into the hemolymph induce spontaneous grooming in naïve cockroaches. Injections of the dopamine receptor agonist SKF 82958 into the SEG also induce prolonged levels of grooming (Weisel-Eichler *et al.* 1999). However, injections of these compounds into stung animals produced no significant grooming compared to sham injection (Weisel-Eichler *et al.* 2002). Interestingly, the time course for recovery of dopamine-induced grooming coincides with the recovery of wind- or touch-evoked escape responses, indicating that one mechanism of action may be responsible for both behavioral phenomena. Dopaminergic signaling and proper dopamine receptor function are important in maintaining normal escape and locomotor behaviors, and dopamine insensitivity in stung animals implies dopamine receptor dysfunction. Inactivation of dopamine receptors by *Ampulex* venom would explain both the block of dopamine-induced grooming in stung animals, and the inability to generate spontaneous movements and escape behaviors.

In this study, I examined the pharmacological profiles of dopamine receptors from *Periplaneta americana* and *Drosophila melanogaster*, and attempted to link dopamine receptor function to venom-induced behaviors. If dopamine is directly involved in grooming behavior, this behavior could theoretically be modified pharmacologically by

treatment with dopamine receptor antagonists and agonists. Dopamine receptors dysfunction may also be the underlying cause of hypokinesia, and cloning these receptors permits the *in vitro* receptor screening of venom components for biologically active compounds (Chapter 4). A D2-like receptor from the American cockroach (called PeaD2R) was sequenced and cloned into an expression vector to establish a pharmacological profile. Additionally, three dopamine receptors from *Drosophila melanogaster* (the D1-like receptors dDA1 (also known as DopR) and DAMB, and the D2-like receptor D2R) were cloned and expressed to identify selective ligands for both types of receptors. We used the bioluminescent properties of the photoprotein aequorin to measure Ca^{2+} release from the endoplasmic reticulum in response to dopamine receptor activation (Park *et al.* 2003; Fichna *et al.* 2006).

Materials and Methods

Insect rearing. *Drosophila melanogaster* (W^{1118}, Bloomington Stock Center #5905, Bloomington, IN) were reared at 25º C under a 12:12 light-dark cycle on standard *Drosophila* medium (University of California, Riverside). *Periplaneta americana* were raised in garbage cans filled with egg cartons on dry dog food (Valutime) and water *ad libitum*. *Ampulex compressa* were raised in plexiglass cages with moderate humidity. Wasps were fed honey and water separately *ad libitum*, and kept under a 16:8 light-dark cycle. For reproduction, adult female cockroaches were placed in the wasp cages along with scintillation vials, 2-4 times per week. Wasps used the scintillation vials as burrows for newly parasitized cockroaches, and vials containing oviposited cockroaches were

moved into a brooding chamber. Freshly eclosed wasps were mixed in cages with older wasps, and experimental wasps (for cockroach envenomation) were selected randomly. Cockroaches were withheld from experimental wasps for at least seven days prior to each behavioral experiment to ensure that each wasp had a full complement of venom components.

Pharmacological Ligands. The following compounds were purchased from Sigma Aldrich (St. Louis, MO): dopamine hydrochloride, tyramine, serotonin creatinine complex, DL-norepinephrine hydrochloride, (±)-octopamine hydrochloride, bromocriptine methanesulfonate salt, (+) butaclamol hydrochloride, (R)-(+)-SCH 23390 hydrochloride, mianserin hydrochloride, (R)-(-) apomorphine hydrochloride hemihydrate, (±)-sulpiride, SKF 82958 hydrobromide, (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide (6,7-ADTN), cis-(Z)-flupenthixol dihydrochloride, 2-ethoxy-1 ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), and (±) SKF 38393 hydrochloride. Fallypride was a kind gift from Dr. Jogeshwar Mukherjee (University of California, Irvine, School of Medicine).

***Periplaneta americana* cDNA cloning and sequencing.** Total RNA was extracted from the brain, subesophageal ganglion and the three thoracic ganglia of adult *Periplaneta americana* using TRIzol (guanidinium thiocyanate/phenol solution for RNA extraction) (Invitrogen, Carlsbad, CA), and was reverse-transcribed into cDNA using the Superscript First Strand Synthesis System for RT-PCR kit (Invitrogen), according to the manufacturer's protocol. A ClustalX (www.clustal.org) alignment of previously identified insect D2-like dopamine receptors (*Drosophila melanogaster, Apis mellifera,*

Bombyx mori, and *Triboleum castaneum*) identified regions of high homology. Degenerate primers were designed from these regions to amplify a segment of the cDNA via the polymerase chain reaction (PCR). Nested PCR with two sets of primers was done to eliminate nonspecific DNA fragments amplified with the degenerate primers. 3' Rapid Amplification of cDNA Ends (RACE) was done via PCR using a poly-T primer ligated to an adapter sequence and a 5' gene-specific primer designed from a region of the cDNA we obtained from degenerate PCR. The 5' end sequence of the cDNA was determined by adding poly-A and poly-C tails to the 3' end of the cDNA via the enzyme terminal deoxynucleotidyl transferase (Promega, Madison, WI). Poly-T and poly-G primers with adapter sequences, along with specific 3' sequence primers from identified DNA, were used to amplify the cDNA segment via nested PCR (Table 3-1). The full-length sequence was amplified via PCR, cloned into the subcloning vector PGEMT (Promega), and subsequently moved into the mammalian expression vector PCDNA3.1+ (Invitrogen) for expression and analysis.

***Drosophila melanogaster* cDNA cloning.** Total RNA was extracted from *Drosophila melanogaster* W^{1118} flies at various stages, and cDNA was generated using Superscript First Strand Synthesis System for RT-PCR kit (Invitrogen), according to the manufacturer's protocol. The transcript sequence of *Drosophila* D2R, dDA1, and DAMB were obtained from FlyBase (www.flybase.org), and specific primers were designed in the 5' and 3' untranslated regions in order to amplify the full-length sequence (Table 3-1). Restriction sites were sometimes engineered into the 5' or 3' end of the cDNAs to facilitate movement into expression vectors. The full length sequences were

subcloned into PGEMT, and subsequently moved into the mammalian expression vector PCDNA3.1+ for expression and analysis. For insect cell expression, dDA1 was cloned into the expression vector pIB/V5-His (Invitrogen).

Phylogenetic analysis. Phylogenetic analysis was performed to assess evolutionary relationships between PeaD2R and other known insect dopamine receptors. Three subtypes of insect dopamine receptors were used for phylogenetic analysis: Dopr1 receptors (D1-like receptors analogous to the *Drosophila* dDA1 receptor), Dopr2 receptors (D1-like receptors analogous to the *Drosophila* DAMB receptor), and D2R receptors (D2-like receptors analogous to *Drosophila* D2R). Dopamine receptor amino acid sequences from *Apis mellifera* (Apm), *Anopheles gambiae* (Ang), *Bombyx mori* (Bom), *Nasonia vitripennis* (Nav), *Triboleum castaneum* (Trc), *Ctenocephalides felis* (Ctf), and *Acyrthosiphon pisum* (Acp) were obtained from the Genbank database. Receptor sequences from *Drosophila melanogaster* (Drm) were obtained from FlyBase (www.flybase.org). Amino acid sequences used for phylogenetic analyses were aligned using T-Coffee (Notredame *et al*. 2000) and tree reconstruction was performed using TREE-PUZZLE v.5.2 (Schmidt *et al*. 2002) with the JTT amino acid substitution model (Jones *et al*. 1992) and among site rate variation accommodated using a discrete gamma shape parameter.

Cell culture and transfection. Chinese hamster ovary (CHO-K1) cells were grown in monolayers in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Invitrogen) containing 10% fetal bovine serum, (Atlanta Biologicals, Atlanta, GA) 1% penicillin/streptomycin, and 1% amphetoricin (fungizone) (Invitrogen) at 37ºC and 5%

CO_2 atmosphere. Chimeric G-proteins allowed G-protein coupled receptors not linked to Gαq to activate phospholipase C, leading to IP_3-mediated calcium release from the endoplasmic reticulum. To establish the specific Gα protein that couples optimally to insect D2-like receptors, three chimeras (Gqi5-HA, Gqo5-HA, and Gqs5-HA, each corresponding to its respective Gα protein; Conklin *et al*. 1993; Conklin *et al*. 1996) were transfected into CHO-K1 cells along with aequorin and a splice variant of *Drosophila* D2R (DD2R-589). A saturating concentration of 5 µM dopamine was used to induce luminescence, using the aequorin cell assay described below.

PeaD2R and *Drosophila* D2R were cotransfected along with plasmids containing the open reading frames for apoaequorin and the chimeric G-protein Gqo5-HA. DAMB was cotransfected along with apoaequorin and the chimeric G-protein Gqs5-HA. dDA1 was transfected only with apoaequorin. All plasmids were transfected using Fugene6 (Roche Applied Science, Indianapolis, IN) with a ratio of 3 µL Fugene/1 µg DNA. Transfected cells were incubated in serum- and antibiotic-free DMEM/F-12 cell culture medium (Invitrogen). All four dopamine receptors were expressed individually in CHO-K1 cells, but dDA1 was also expressed in insect cells. Hi5 insect cells (*Trichoplusia ni*) (Invitrogen) were grown in monolayers in TMN-FH cell culture media (Sigma Aldrich) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphetoricin at 27ºC. dDA1 was cotransfected along with apoaequorin using Fugene6 as described above, and incubated into serum-free and antibiotic-free media.

Aequorin Luminescence Assay. CHO-K1 cells were harvested 24 hours after transfection and incubated at room temperature in BSA medium (DMEM/F-12 containing

1% bovine serum albumin and 1% penicillin/streptomycin) with 5 µM of the prosthetic luminophore coelenterazine h (Anaspec, Fremont, CA) for 2.5 hours. Cells were then diluted to a concentration of 5 x 10^6 cells/mL, and incubated for an additional 45-60 minutes. Hi5 cells were incubated with 5 µM coelenterazine h while attached to the culture flask in order to avoid cell death from mixing while in suspension. Pharmacological ligands were prepared fresh immediately prior to analysis. All compounds were initially prepared at concentration of 1 mM or 10 mM, and subsequently diluted in BSA/TNM-FH medium (depending on cell type). All the biogenic amines, quinpirole, and SCH 23390 were dissolved in BSA medium/TNM-FH medium. Flupenthixol, apomorphine, SKF 38393, and SKF 82958 were initially dissolved in water. Bromocriptine, sulpiride, fallypride, butaclamol, and mianserin were dissolved in ethanol. 6,7-ADTN was dissolved in water with 0.1% sodium metabisulfite. EEDQ was dissolved in acetone. Fifty µL of each ligand solution were pipetted into the wells of a 96-well Costar round bottom white polystyrene luminescence plate (Corning, Lowell, MA). For antagonist assays, 25 µL of antagonist solution was mixed with 25 µL of dopamine solution (final concentration was the EC_{80} concentration for each receptor, determined from my dose response curves). A Berthold TriStar LB 941 luminometer (Berthold, Oak Ridge, TN) pumped 50 µL of cell suspension into each well, and recorded luminescence levels for 20 seconds with a sampling rate of 10 readings/second. Each compound measurement was performed in quadruplicate and averaged. The average background luminescence, determined by measuring luminescence from cells in media with no ligand, was subtracted in order to only measure luminescence generated by

ligand-receptor interactions. Dose response curves were plotted as the percent of relative luminescence units (RLU) normalized to the maximum response for each ligand. EC_{50}/IC_{50}s for dose-response curves were determined using Origin 6.1 graphing software (Originlab, Northampton, MA).

Behavioral Analysis. Sulpiride was prepared at a concentration of 10 mM in 10% aqueous acetic acid, and diluted to a final concentration of 100 µM with phosphate-buffered saline (PBS, 0.01 M phosphate, 0.0027 M KCl, 0.137 M NaCl, pH 7.4). Bromocriptine was prepared at a concentration of 10 mM in 1 % acetic acid, and diluted to a final concentration of 1 mM in PBS. SCH 23390 and flupenthixol were prepared in PBS and diluted to a final concentration of 100 µM. All chemicals were prepared immediately before injection. Ten µL of each solution were injected into the abdominal hemolymph of the cockroach, under the third or fourth abdominal sternite. Approximately 1 hour after injection, wasps were allowed to perform the full repertoire of stings into the cockroach central nervous system. The cockroach was separated from the wasp as soon as the stinging sequence was finished, and immediately placed into a small chamber for grooming analysis. Animals were filmed, and the duration of grooming within 1 hour after the initial onset of grooming was measured. All experiments were performed on adult female cockroaches.

Results

***Periplaneta americana* D2-like receptor sequence analysis.** The full-length cDNA encoding a D2-like dopamine receptor (PeaD2R) was cloned from the head and thoracic

ganglia of the American cockroach. The sequenced cDNA was 1987 base pairs, and the open reading frame encoded a 533 amino acid protein. A prediction of transmembrane helices (TMHMM, http://www.cbs.dtu.dk/services/TMHMM/) indicated that the amino acid sequence contains seven transmembrane domains with a long third intracellular loop, a common feature of D2-like dopamine receptors. The amino acid sequence contained specific signature amino acid motifs which are critical for G-protein coupling and activation, including a run of charged residues (lysine and arginine) in the C-terminal end of the third intracellular domain, aspartate-arginine (DR) at the third transmembrane/second intracellular domain junction (Wess 1997), and the conserved asparagine-proline-XX-tyrosine (NPXXY) motif in the seventh transmembrane domain (where X is any amino acid) (Gales *et al.* 2000). There is a valine-threonine-XX-isoleucine-leucine (VTIL) motif found at the third extracellular domain/seventh transmembrane domain junction. A similar motif (VTIV, where valine replaces the carboxyl leucine) is also found in the seventh transmembrane region of the D2-like receptor of *Apis mellifera* (AMDop3) (Beggs *et al.* 2005). It has been reported that this motif is important for receptor coupling to Gαi/o subunits on the C-terminal end of G-proteins, and is found at the third intracellular/sixth transmembrane domain junction in the m2 muscarinic receptor (Wess 1997; Kostenis *et al.* 1998). However, it is unlikely that this particular motif is involved in G-protein coupling of PeaD2R because it is predicted to be found in an extracellular/transmembrane region of the receptor (TMHMM Server, version 2.0. http://www.cbs.dtu.dk/services/TMHMM/), and not in position for intracellular coupling.

BLAST analysis of PeaD2R against sequences in the Genbank database revealed regions with high levels of homology to other insect D2-like dopamine receptors, with amino acid conservation normally greater than 60% (64% for *Apis mellifera*, 62% for *Drosophila melanogaster*). An amino acid alignment with other D2-like receptors showed specific regions of homology that are well conserved among the insects, and partially conserved when compared with human D2R (Figure 3-3). The phylogenetic analysis showed that PeaD2R is evolutionarily grouped with other insect D2-like receptors, and distinct from the D1-like receptor subtypes (Figure 3-4). The two subtypes of D1-like receptors were grouped into distinct families, but appear to share a common ancestor.

Insect D2-like receptors couple with Gαo to initiate intracellular signaling. Receptor activity was measured using a luminescence assay that detected Ca^{2+} released in the cytosol. The 21 kDa photoprotein apoaequorin, isolated from the jellyfish *Aequora victoria*, is a sensitive reporter of Ca^{2+} levels. Apoaequorin forms a complex (aequorin) with the luminophore cofactor coelenterazine, which has two binding sites for Ca^{2+} (Shimomura 1995). Ca^{2+} binding causes an oxidation reaction of coelenterazine, which produces apoaequorin, coelenteramide, carbon dioxide, and light with a λ_{max} at 469 nm (Stables *et al.* 1997). Receptor activity is correlated to light emission, thus with increasing ligand concentrations, luminescence levels increase until receptor saturation is reached.

It has been previously reported that insect D2-like receptors follow the Gαi/o signaling pathway (Hearn *et al.* 2002; Clark *et al.* 2007; Draper *et al.* 2007), leading to

reduced activity of adenylate cyclase, and lower levels of cAMP. Chimeric G-proteins allow G-protein coupled receptors normally coupled to Gs, Gi, or Go, to initiate the Gq downstream signaling cascade, and have been used previously to measure intracellular signaling in response to dopamine receptor binding (Hearn *et al.* 2002). This pathway activates phospholipase C (PLC), which liberates inositol triphosphate (IP_3) from the plasma membrane. IP_3 subsequently binds to receptors on the endoplasmic reticulum, leading to Ca^{2+} release, which is detected by the aequorin complex. Three different chimeric G-proteins, Gqs5-HA, Gqi5-HA, and Gqo5-HA (Conklin *et al.* 1993; Conklin *et al.* 1996), were tested in CHO-K1 cells to establish the specific Gα protein that couples optimally to insect D2-like receptors. All three chimeric G-proteins facilitated dopamine-induced luminescence within cells, but the signal was strongest from cells expressing the Gqo5-HA chimera (Fig. 3-4). Gqi5-HA induced luminescence levels that were approximately three times lower than Gqo5-HA, and the luminescence levels from cells expressing Gqs5-HA were the lowest.

PeaD2R biogenic amine pharmacology. Previous studies measured cAMP accumulation in response to dopamine receptor binding in *Drosophila melanogaster* (Sugamori *et al*. 1995; Feng *et al*. 1996; Han *et al*. 1996), *Apis mellifera* (Mustard *et al*. 2003; Beggs *et al*. 2005), *Ctenocephalides felis* (Gerber *et al*. 2006), and *Bombyx mori* (Ohta *et al.* 2009), where cAMP measurement occurred 10-30 minutes after treatment with ligands. In this study, we used an aequorin-based luminescence assay to measure receptor binding (Park *et al.* 2003; Fichna *et al.* 2006). The chimeric G-protein Gqo5-

HA was utilized to initiate the Gq downstream signaling pathway, leading to intracellular calcium mobilization from the endoplasmic reticulum.

Insect dopamine receptors are activated by a variety of biogenic amines other than dopamine (Han *et al.* 1996; Blenau *et al.* 1998; Hearn *et al.* 2002; Beggs *et al.* 2005). Dose-response curves for dopamine, serotonin, tyramine, norepinephrine, and octopamine were generated using the aequorin-based luminescence assay (Figure 3-6). Dopamine was the most potent ligand (EC_{50} = 1.33 nM), followed by norepinephrine (EC_{50} = 182 nM). Serotonin (EC_{50} = 928 nM) and tyramine (EC_{50} = 1140 nM) also show considerable agonist activity at the receptor, while octopamine was the weakest amine tested (EC_{50} = 38300 nM).

PeaD2R synthetic agonist pharmacology. The pharmacology of dopamine receptors has been well established in mammalian systems, but insect dopamine receptors have not been characterized as thoroughly. A number of synthetic dopamine receptor agonists were tested for receptor activity. The nonselective dopamine agonist 6,7-ADTN was the most potent agonist tested, with an EC_{50} (6.12 nM) that was close to the EC_{50} of dopamine (1.33 nM). The D2-selective compounds bromocriptine (EC_{50} = 153 nM) and quinpirole (EC_{50} = 21600 nM), in addition to the nonselective agonist apomorphine also elicited significant luminescence responses. Surprisingly, the D1-selective compounds SKF 82958 (EC50 = 621 nM) and SKF 38393 (EC_{50} = 1818 nM) activated the D2-like receptor as well (Figure 3-7).

PeaD2R antagonist pharmacology. Dopamine receptor antagonists were coapplied with 5 nM dopamine (EC_{80} value) to determine antagonist potencies. The mammalian

D1-like antagonist SCH-23390 and the mammalian D2-like antagonist sulpiride had nearly identical inhibition curves (IC_{50}s of 1340 nM and 1410 nM respectively). Fallypride, a D2-like antagonist (Buchsbaum *et al.* 2006), and the nonspecific antagonist flupenthixol also antagonized dopamine binding, albeit with higher IC_{50}s (9930 nM and 28500 nM respectively) (Figure 3-8). The dopamine receptor antagonists EEDQ, and butaclamol, and the octopamine/serotonin receptor antagonist mianserin had no observable antagonistic effects.

***Drosophila melanogaster* D2R pharmacology.** To compare PeaD2R receptor pharmacology with a previously characterized D2-like receptor, a splice variant of the *Drosophila* D2-like receptor, DD2R-589, was cloned and expressed in CHO-K1 cells. The pharmacological profile of the DD2R-589 receptor closely resembled that of the D2-like cockroach receptor, with dopamine being the most potent ligand (EC_{50} = 21.9 nM) followed by norepinephrine (EC_{50} = 893 nM), serotonin (EC_{50} = 2490 nM), tyramine (EC_{50} = 7520), and octopamine (EC_{50} = 136000 nM) (Figure 3-9). 6,7-ADTN (EC_{50} = 62.6 nM) and apomorphine (EC_{50} = 85.5 nM) were the most potent synthetic agonists tested, whereas bromocriptine (EC_{50} = 612 nM), SKF 82958 (EC_{50} = 4700 nM), SKF 38393 (EC_{50} = 14200), and quinpirole (EC_{50} = 165000) all generated significant levels of luminescence (Figure 3-10). SCH-23390 and sulpiride were equally effective at antagonizing dopamine receptors (IC_{50}s of 21600 nM and 21000 nM respectively), and slightly more effective than flupenthixol (IC_{50} = 49300 nM) (Figure 3-11). Fallypride was relatively ineffective (IC_{50} = 247000 nM). EEDQ, butaclamol, and mianserin had no observable effect, and did not suppress dopamine-induced luminescence.

***Drosophila* DAMB receptor pharmacology.** Dopamine was the best ligand, with an EC_{50} of 2.72 nM. 6,7-ADTN was nearly as potent as dopamine, with an EC_{50} of 2.83 nM. Apomorphine (EC_{50} = 618 nM), SKF 82958 (EC_{50} = 1810 nM), and SKF 38393 (EC_{50} = 3309 nM) also demonstrated agonist activity (Figure 3-12). Surprisingly, the D2-selective compound quinpirole induced receptor activity, albeit with a lower potency than the aforementioned agonists (EC_{50} = 6480 nM). The D2-selective compound bromocriptine had no observable effect on receptor activation. It should be noted that although the Gqs chimeric G-protein was used to facilitate calcium mobilization within the cell in order to generate luminescence, luminescence was also generated in the absence of chimeric G-protein. The EC_{50} for dopamine in cells not expressing the chimera was 2.79 nM (data not shown), indicating that the presence of the chimera did not appear to overtly affect receptor pharmacology. However, it has been previously demonstrated that different synthetic ligands at DAMB can selectively trigger different signaling cascades, leading to varying degrees of cAMP accumulation and intracellular calcium mobilization (Reale *et al.* 1997). Our luminescence assay only detects calcium mobilization, so we utilized the chimeric G-protein to ensure that the synthetic ligands we applied led to calcium mobilization, and not just adenylate cyclase activation. Although there is no direct evidence that the chimera had any impact on calcium mobilization, compounds that failed to generate calcium currents in *Xenopus laevi* oocytes (SKF 38393 and quinpirole) (Feng *et al.* 1996) generated luminescence in our assay, indicating that these compounds did induce calcium mobilization. However, SKF 38393 (10 µM) and quinpirole (10 µM) also failed to stimulate cAMP accumulation in *Xenopus* oocytes after

incubation for 30 minutes (Reale *et al.* 1997), suggesting that these compounds do not initiate the Gs or Gi/o signaling pathways. Alternatively, it is possible that the experimental methods used in that study were not optimal for those particular agonists, because we were able to get receptor responses with both compounds using the aequorin-based assay.

The nonselective antagonist SCH 23390 was the best antagonist, with an IC_{50} of 3310 nM. Flupenthixol and mianserin partially suppressed dopamine induced luminescence (to 46 % and 36.1 % of the maximum signal respectively), but full suppression was not obtained, even at concentrations of 100000 nM (Figure 3-13). The antagonists EEDQ, butaclamol, fallypride, and sulpiride had no observable effect on receptor activity.

***Drosophila* dDA1 receptor pharmacology.** dDA1 was initially expressed in CHO-K1 cells for pharmacological profiling, and the dose-response curve for dopamine was established (EC_{50} = 6730 nM) (Figure 3-14). However, it was reported previously that this receptor was 10-fold less sensitive to dopamine when expressed in mammalian cells, compared to expression in insect cells (Sugamori *et al.* 1995). dDA1 was subsequently expressed in insect Hi5 cells, which increased receptor sensitivity to dopamine 10-fold (EC_{50} = 652 nM), confirming the previous findings. All subsequent pharmacological profiles for dDA1 were generated using Hi5 cells.

6,7-ADTN was an effective agonist (EC_{50} value of 2770 nM) (Figure 3-15), but apomorphine, SKF 89528, SKF 38393, quinpirole, and bromocriptine all failed to activate the receptor. SCH 23390 (IC_{50} = 424 nM), mianserin (IC_{50} = 1410 nM),

flupenthixol (IC_{50} = 4470 nM), and butaclamol (IC_{50} = 13900 nM) all inhibited dopamine-induced luminescence (Figure 3-16). Even though SCH 23390 and mianserin were able to almost entirely suppress dopamine-induced luminescence at 10 µM, their response curves were different. Flupenthixol and butaclamol were also effective antagonists, but the full luminescence response was not attained at low doses of the antagonists, appearing to plateau at 85-90% of the control value. This suggests that these drugs may be inhibiting receptor activity noncompetitively. Sulpiride, EEDQ, and fallypride all failed to block dopamine receptor activity.

***Ampulex compressa* Venom-Induced Grooming Behavior.** Wasp stings into the head cavity of the cockroach induce compulsive grooming. It had been postulated that dopamine receptor activation is responsible for sting-induced grooming behavior (Weisel-Eichler *et al*. 1999). To test this, we treated cockroaches with various compounds that display biological activity at dopamine receptors. Pretreatment with 1 nmole of the D2-selective antagonist sulpiride 1 hour prior to the sting slightly, but significantly, reduced the grooming duration induced by the head sting. However, pretreatment with the nonselective dopamine receptor antagonists SCH 23390 (1 nmole) and flupenthixol (1 nmole), or the D2-like agonist bromocriptine (10 nmoles) had no significant effect on grooming duration (Figure 3-17). This indicates that only selective inhibition of D2-like receptors reduces grooming duration, whereas inhibition of both D1- and D2-like receptors simultaneously, and pretreatment with a D2-selective agonist, had no observable effect.

Discussion

Dopaminergic signaling in insects is essential for maintenance of many physiological functions. Envenomation by the parasitoid wasp *Ampulex compressa* induces symptoms in cockroaches that resemble behavioral effects when dopaminergic signaling is disrupted, including reductions in spontaneous locomotion (Pendleton *et al.* 2002; Draper *et al.* 2007) and akinesia (Yellman *et al.* 1997). The mechanism of action of *Ampulex* venom is currently unknown, but one possibility is that the venom alters dopaminergic signaling in the central nervous system.

To test this hypothesis, I cloned a dopamine receptor from the American cockroach, *Periplaneta americana*. We elected to clone the D2-like receptor, as opposed to D1-like receptors, because D2-like receptors have been intimately linked to locomotion in both mammals and insects. D2 knockout mice displayed significant reductions in spontaneous locomotion (Baik *et al.* 1995; Kelly *et al.* 1998), whereas D1 knockout mice were hyperactive (Xu *et al.* 1994), or showed no significant difference from wild-type mice (Drago *et al.* 1994). Similar behaviors were observed in *Drosophila*, where D2R RNAi flies displayed reduced levels of locomotion (Draper *et al.* 2007).

Analysis of the amino acid sequence of PeaD2R revealed many characteristics of rhodopsin-like G-protein coupled receptors, and similarities with previously sequenced insect D2-like receptors. There is approximately 60-64% homology between PeaD2R and other insect D2-like dopamine receptors (*Drosophila melanogaster*, *Apis mellifera*, and *Triboleum castaneum*), indicating that this receptor is relatively well conserved, even

across different insect orders. The phylogenetic analysis demonstrated that PeaD2R was evolutionarily similar to other insect D2-like receptors, and quite distinct from D1-like receptors. The VLIT motif common to Gi/o linked GPCR's was detected in PeaD2R, and slight variants were also present in the D2-like receptor of *Apis mellifera* (Beggs *et al.* 2005) and *Drosophila melanogaster* (Hearn *et al.* 2002). This motif was reported to couple the G-protein to the receptor (Wess 1997; Kostenis *et al.* 1998), but in PeaD2R, the motif was predicted to be found in the third extracellular/seventh transmembrane domain of the receptor, as opposed to the interior side of the cell. This indicates that this motif is most likely not involved in receptor coupling, but may serve some other function.

Dopamine receptor activation was measured using an aequorin-based cell luminescence assay. CHO-K1 cells transfected with an individual dopamine receptor, aequorin, and a chimeric G-protein, were exposed to dopamine, which triggered an intracellular signaling cascade, ultimately leading to calcium release from the endoplasmic reticulum. In the presence of coelenterazine h, calcium activated aequorin, leading to a flash of luminescence at 469 nm, which was measured with a luminometer (Park *et al.* 2003; Fichna *et al.* 2006). This methodology allowed us to record receptor activity in real-time, eliminating the need to incubate cells for extended periods with ligands, or artificially raise cAMP levels with forskolin when measuring adenylate cyclase inhibition.

D2-like receptors typically initiate the Gi/o signaling cascade, leading to decreased levels of cAMP (Enjalbert *et al.* 1983). In this study, it was demonstrated that insect D2-like receptors preferentially couple to Gαo by measuring signal activation

through different chimeric G-proteins. This was not an unexpected result, because D2 dopamine receptors in the central nervous system of mice are coupled to Go (Jiang *et al.* 2001). The Gqo5-HA chimera effectively coupled receptor activation to calcium mobilization. The amount of luminescence generated was directly proportional to the concentration of ligand applied, allowing us to quickly establish pharmacological profiles and dose-response relationships.

Dopamine was the most potent ligand for PeaD2R. However, significant levels of activity were observed when many other biogenic amines and synthetic agonists were applied. Receptor activation was not only initiated by mammalian D2-like selective agonists (bromocriptine and quinpirole) but also by mammalian D1-like selective agonists (SKF 82958 and SKF 38393) as well. This demonstrates that insect receptor pharmacology can differ considerably from that of their mammalian counterparts.

The pharmacology of PeaD2R was compared against a previously profiled insect D2-like receptor, *Drosophila* D2R, to determine if the receptors behave comparably. The pharmacology of the *Drosophila* D2R-589 splice variant was very similar to PeaD2R's profile, except that all the ligands were 2-8 times less potent. Similarities in protein sequence and functional profiles strongly suggest that PeaD2R is a D2-like dopamine receptor.

The analysis of *Drosophila* D2R-589 pharmacology revealed some differences with previous studies on this receptor, although the general trends remain relatively consistent. Hearn *et al.* initially cloned and profiled three splice variants of this receptor using an *in vitro* luciferase assay in HEK 293 cells. The aequorin-based luminescence

assay employed in this study appears to be more sensitive, because the EC_{50} for dopamine established using this method (1.39 nM) was 400 times lower than the published value (500 nM) (Hearn *et al.* 2002). However, the EC_{50}s for the other biogenic amines were comparable, although we found norepinephrine to be more potent than serotonin, whereas Hearn *et al.* found the opposite.

It was also reported that bromocriptine was two orders of magnitude more potent than dopamine, and that butaclamol acted as a weak antagonist (Hearn *et al.* 2002). We found that bromocriptine is a good agonist, but is approximately 30 times less potent than dopamine. In addition, we did not see any antagonistic properties of butaclamol. These discrepancies may be the result of using a splice variant (*Drosophila* D2R-589) that was not previously profiled, but it is more likely due to the way receptor activity was measured. Hearn *et al.* incubated cells expressing D2R with ligand for 3 hours, and subsequently measured luciferase activity. This long incubation time may have led to oxidation/degradation of ligands, altering perceived ligand efficacy, or somehow affected the signaling pathways. We monitored receptor activation for 20 seconds, watching receptor activity via the flash luminescence response in real time. This methodology allowed us to measure receptor activation reliably and reproducibly without additional concern of incubation time.

My results show that insect dopamine receptors display pharmacological profiles distinct from mammalian dopamine receptors. Distinguishing the pharmacology of PeaD2R and *Drosophila* D2R from the *Drosophila* D1-like receptors allowed us to determine which agonists and antagonists act specifically at each receptor subtype. It

was important to experimentally establish the receptor targets each drug acts upon in order to ensure that the conclusions based on the experimental evidence were accurate, particularly when investigating behavioral effects *in vivo.*

dDA1 and DAMB are D1-like dopamine receptors, whose pharmacology has been profiled previously (Sugamori *et al.* 1995; Feng *et al.* 1996; Han *et al.* 1996; Reale *et al.* 1997; Hearn *et al.* 2002). We found that the EC_{50} of dopamine at DAMB (2.42 nM) was comparable to the EC_{50} at PeaD2R (1.39 nM), and that most of the agonists tested activated these two receptors. Bromocriptine, a potent agonist of PeaD2R and *Drosophila* D2R, was a notable exception because of its lack of activity at DAMB. The EC_{50} of dDA1 expressed in Hi5 cells (652 nM) was considerably larger than the EC_{50}s of the other receptors, and the majority of synthetic agonists tested failed to elicit receptor activity. 6,7-ADTN was the only synthetic compound that activated the receptor.

The mammalian D1-selective agonists SKF 38393 and SKF 82958 induced D1-like receptor activity at DAMB (EC_{50}s of 3310 nM and 1810 nM respectively) and D2-like receptor activity in insects (PeaD2R EC_{50}s of 1818 nM and 660 nM respectively). Surprisingly, the ligands appeared to be more potent at insect D2-like receptors than D1-like receptors (SKF 89528 and SKF 38393 failed to activate dDA1 entirely), indicating that these ligands are not appropriate for selectively studying D1-like receptor activity in insects. In addition, the D2-selective drug quinpirole had considerable activity at DAMB (EC_{50} = 6480 nM), which was considerably more potent than at PeaD2R (EC_{50} = 21600 nM). This indicates that despite quinpirole's previous use as a D2-selective dopamine

receptor agonist in insects (Yellman *et al.* 1997; Rosenberg *et al.* 2007), it may not be the ideal choice for study of this particular receptor.

The receptor subtypes also displayed different profiles for antagonists. Of all the antagonists tested, SCH 23390 was the best nonselective antagonist (IC_{50}s of 424 nM, 1340 nM, and 3310 nM for dDA1, PeaD2R, and DAMB respectively). Sulpiride and fallypride were selective for the D2-like receptor, and had no observable effect on the D1-like receptors. Conversely, mianserin, an antagonist of octopamine receptors (Evans 1981; Bischof *et al.* 2004) and serotonin receptors (Stewart *et al.* 1989) inhibited dopamine-induced activity at dDA1 and DAMB, with no observable effect at the D2-like receptors. Mianserin's efficacy was not entirely surprising, because BLAST analyses of the amino acid sequences show that DAMB and dDA1 have considerable levels of homology with both dopamine receptors and other biogenic amine receptors as well (Sugamori *et al.* 1995; Feng *et al.* 1996). Butaclamol, reported to have weak antagonist activity at *Drosophila* D2R (Hearn *et al.* 2002), showed antagonism only at dDA1.

It has been reported previously that flupenthixol significantly reduces dopamine-induced receptor activity in both D1- and D2-like receptors (Sugamori *et al.* 1995; Feng *et al.* 1996; Han *et al.* 1996; Hearn *et al.* 2002). We also observed receptor antagonism in the presence of flupenthixol, although it appeared to be considerably weaker than what had been previously reported. We were only able to get full suppression of receptor activity at dDA1. Dopamine was able to induce receptor activity at DAMB or PeaD2R, even when antagonist concentrations of 100 µM were applied. This may be due to the fact that flupenthixol was treated as a competitive agonist, and we looked for suppression

immediately after ligand application, whereas other studies allowed flupenthixol to incubate with cells for longer periods of time (Sugamori *et al.* 1995; Feng *et al.* 1996; Han *et al.* 1996). At high concentrations, flupenthixol acts noncompetitively (Breward *et al.* 1980), so perhaps its full antagonistic potential was not achieved in the time-frame used for this study.

It was previously proposed that *Ampulex* venom-induced grooming in cockroaches is caused by the activation of dopamine receptors (Weisel-Eichler *et al.* 1999). We wanted to determine if the grooming behavior elicited upon envenomation can be altered by pretreating the animals with compounds that act on dopamine receptors. It was previously reported that flupenthixol pretreatment (200-600 nmoles) dramatically reduced sting-induced grooming behavior (Weisel-Eichler *et al.* 1999). We injected 1 nmole into the cockroach hemolymph and saw no observable difference when compared to animals injected with buffer only. It was somewhat surprising that 1 nmole of flupenthixol had no observable effect, but perhaps an extremely high dose is needed for flupenthixol to penetrate the perineurium and gain access to the central nervous system.

The D2-selective antagonist sulpiride was the only compound tested that had an observable effect on grooming duration (1 nmole of flupenthixol, SCH 23390, or 10 nmoles of bromocriptine neither reduced nor enhanced venom-induced grooming). Sulpiride pretreatment caused a slight, but significant, reduction in grooming duration, reducing the overall duration by approximately five minutes. Interestingly, the nonselective antagonists SCH 23390 and flupenthixol had no effect on grooming duration. This indicates that activation of specific receptor subtypes can differentially

affect grooming behaviors. This has been observed in mammalian systems, where D1-like and D2-like activation result in differences in stereotypic behaviors (Berridge *et al.* 2000a; Berridge *et al*. 2000b). However, it is possible that sufficient doses of SCH 23390, flupenthixol, and bromocriptine required for biological activity did not reach the central nervous system, due to degradation or metabolism. The relatively weak effect of sulpiride may also be due to difficulty reaching the central nervous system. Sulpiride is hydrophilic (Alam *et al.* 1979), and may weakly penetrate the insect blood brain barrier when injected into the hemolymph, thus the actual dose at the receptors within the central nervous system may be much less than the injected dose.

Establishing the pharmacological profiles of insect dopamine receptors has provided useful information which we can utilize to study the roles of each receptor subtype in insect physiology. Our data support, albeit weakly, the hypothesis that dopaminergic systems are involved in sting-induced grooming behavior (Weisel-Eichler *et al*. 1999). However, the roles of dopamine receptors in sting-induced hypokinesia have yet to be firmly established. The impact of *Ampulex* venom on dopamine receptor function, and an investigation of other functional properties of the venom, will be the subject of the next chapter.

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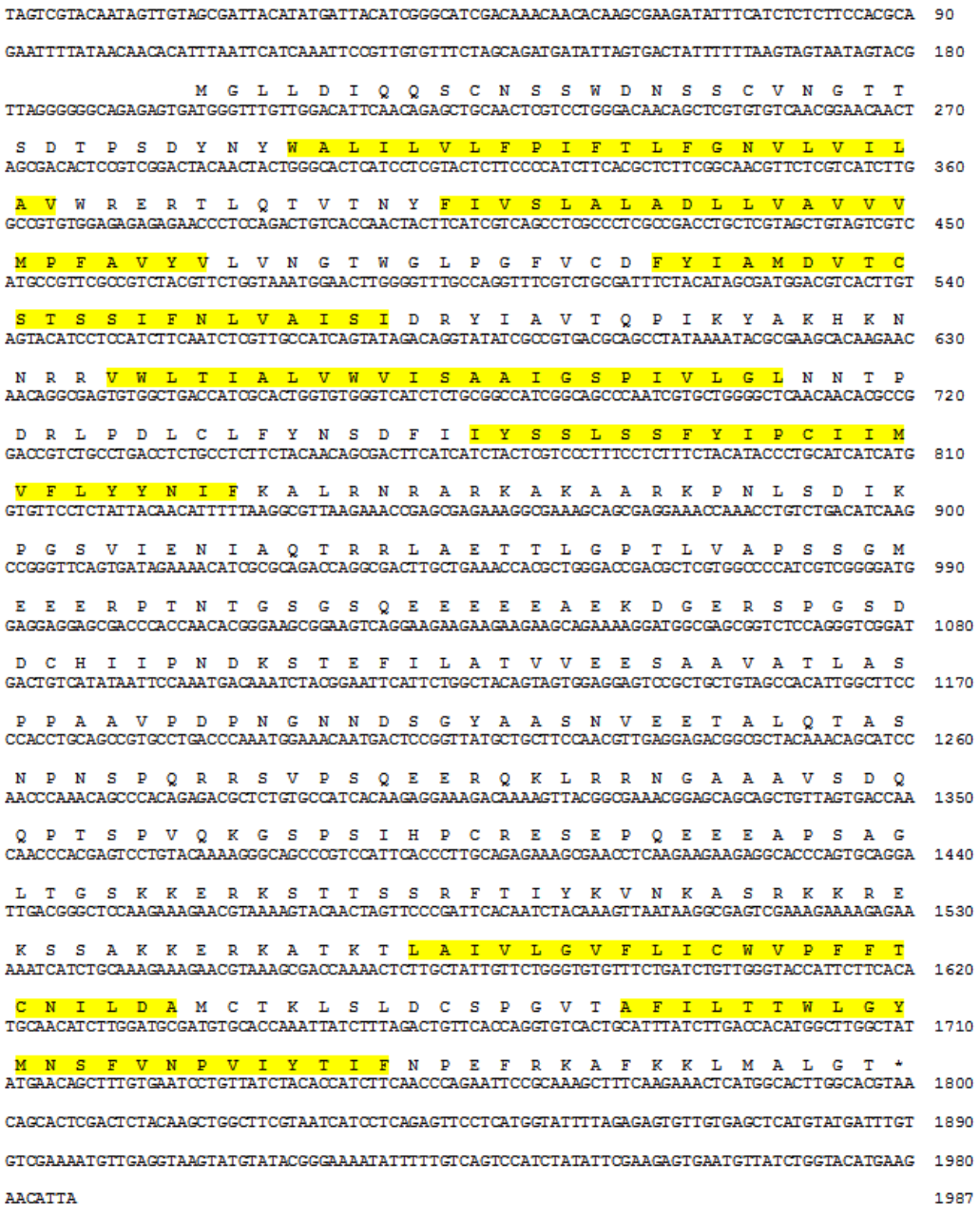
Table 3-1. Oligonucleotide primers for RT-PCR amplification of the D2-like receptor from *Periplaneta americana* (PeaD2R), and the three dopamine receptors from *Drosophila melanogaster* (dDA1, DAMB, D2R).

Primer	Function	Nucleotide Sequence 5' → 3'
1671 F5	Degenerate, PeaD2R	GTNTAYTTYCARGTNAAYGG
1672 F6	Degenerate, PeaD2R	ATHTTYAAYYTIGTNGCNAT
1675 R6	Degenerate, PeaD2R	TCNGGRTTRAADATNGTRTA
1676 R7	Degenerate, PeaD2R	TTRTAICCNARCCANGTNGT
1707 3' F1	3' RACE, PeaD2R	GCGAAGCACAAGAACAACAG
1709 3' F3	3' RACE, PeaD2R	GCAAAGAAAGAACGTAAAGC
K40	3' & 5' RACE, PeaD2R	GACTCGAGTCGACATCGATTTTTTT-TTTTTTTTTT
K41	3' & 5' RACE, PeaD2R	GACTCGAGTCGACATCG
1761 5' AP	5' RACE, PeaD2R	GACTCGAGTCGACATCGACCCCCC-CCCCCCCCCCC
1747 5' R8	5'RACE, PeaD2R	CACGGCGATATACCTGTCTA
1748 5' R9	5' RACE, PeaD2R	ATGGCAACGAGATTGAAGAT
1752 5' R13	5' RACE, PeaD2R	GAGGATGAGTGCCCAGTAGT
1753 5' R14	5' RACE, PeaD2R	GCCGAAGAGCGTGAAGATGG
1745 F1	Full, *Drosophila* D2R	ATAGAATTCATAGAGACGCTGGGC-AATCGAAC
1746 R1	Full, *Drosophila* D2R	ATAGCGGCCGCATATAATCACCCC-ATGTGCATGA
1888 F2	Full, dDA1	AAGGTACCATACCATCCACCAACCA TCCATTC
1885 R1	Full, dDA1	CTCCACCGCCAGTGAGATTC
1958 F1	Full, DAMB	AAGGTACCATAAATTGGAACAGGA-ACAGAATCAGGC
1979 R1	Full, DAMB	AGCTGCCGTCACATGAGCGT

Figure 3-1. Nucleotide and amino acid sequence of the D2-like dopamine receptor from the American cockroach, *Periplaneta americana*. The open reading frame is 1599 base pairs long, and encodes a protein that is 533 amino acids in length. Highlighted regions are predicted transmembrane domains (TMHMM Server, V 2.0. http://www.cbs.dtu.dk/services/TMHMM/).



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GAATTTTATAACAACACATTTAATTCATCAAATTCCGTTGTGTTTCTAGCAGATGATATTAGTGACTATTTTTTAAGTAGTAATAGTACG 180

M G L L D I Q Q S C N S S W D N S S C V N G T T
TTAGGGGGGCAGAGAGTGATGGGTTTGTTGGACATTCAACAGAGCTGCAACTCGTCCTGGGACAACAGCTCGTGTGTCAACGGAACAACT 270

S D T P S D Y N Y W A L I L V L F P I F T L F G N V L V I L
AGCGACACTCCGTCGGACTACAACTACTGGGCACTCATCCTCGTACTCTTCCCCATCTTCACGCTCTTCGGCAACGTTCTCGTCATCTTG 360

A V W R E R T L Q T V T N Y F I V S L A L A D L L V A V V V
GCCGTGTGGAGAGAGAGAACCCTCCAGACTGTCACCAACTACTTCATCGTCAGCCTCGCCCTCGCCGACCTGCTCGTAGCTGTAGTCGTC 450

M P F A V Y V L V N G T W G L P G F V C D F Y I A M D V T C
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S T S S I F N L V A I S I D R Y I A V T Q P I K Y A K H K N
AGTACATCCTCCATCTTCAATCTCGTTGCCATCAGTATAGACAGGTATATCGCCGTGACGCAGCCTATAAAATACGCGAAGCACAAGAAC 630

N R R V W L T I A L V W V I S A A I G S P I V L G L N N T P
AACAGGCGAGTGTGGCTGACCATCGCACTGGTGTGGGTCATCTCTGCGGCCATCGGCAGCCCAATCGTGCTGGGGCTCAACAACACGCCG 720

D R L P D L C L F Y N S D F I I Y S S L S S F Y I P C I I M
GACCGTCTGCCTGACCTCTGCCTCTTCTACAACAGCGACTTCATCATCTACTCGTCCCTTTCCTCTTTCTACATACCCTGCATCATCATG 810

V F L Y Y N I F K A L R N R A R K A K A A R K P N L S D I K
GTGTTCCTCTATTACAACATTTTTAAGGCGTTAAGAAACCGAGCGAGAAAGGCGAAAGCAGCGAGGAAACCAAACCTGTCTGACATCAAG 900

P G S V I E N I A Q T R R L A E T T L G P T L V A P S S G M
CCGGGTTCAGTGATAGAAAACATCGCGCAGACCAGGCGACTTGCTGAAACCACGCTGGGACCGACGCTCGTGGCCCCATCGTCGGGGATG 990

E E E R P T N T G S G S Q E E E E E A E K D G E R S P G S D
GAGGAGGAGCGACCCACCAACACGGGAAGCGGAAGTCAGGAAGAAGAAGAAGAAGCAGAAAAGGATGGCGAGCGGTCTCCAGGGTCGGAT 1080

D C H I I P N D K S T E F I L A T V V E E S A A V A T L A S
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P P A A V P D P N G N N D S G Y A A S N V E E T A L Q T A S
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Q P T S P V Q K G S P S I H P C R E S E P Q E E E A P S A G
CAACCCACGAGTCCTGTACAAAAGGGCAGCCCGTCCATTCACCCTTGCAGAGAAAGCGAACCTCAAGAAGAAGAGGCACCCAGTGCAGGA 1440

L T G S K K E R K S T T S S R F T I Y K V N K A S R K K R E
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K S S A K K E R K A T K T L A I V L G V F L I C W V P F F T
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C N I L D A M C T K L S L D C S P G V T A F I L T T W L G Y
TGCAACATCTTGGATGCGATGTGCACCAAATTATCTTTAGACTGTTCACCAGGTGTCACTGCATTTATCTTGACCACATGGCTTGGCTAT 1710

M N S F V N P V I Y T I F N P E F R K A F K K L M A L G T *
ATGAACAGCTTTGTGAATCCTGTTATCTACACCATCTTCAACCCAGAATTCCGCAAAGCTTTCAAGAAACTCATGGCACTTGGCACGTAA 1800

CAGCACTCGACTCTACAAGCTGGCTTCGTAATCATCCTCAGAGTTCCTCATGGTATTTTAGAGAGTGTTGTGAGCTCATGTATGATTTGT 1890

GTCGAAAATGTTGAGGTAAGTATGTATACGGGAAAATATTTTTGTCAGTCCATCTATATTCGAAGAGTGAATGTTATCTGGTACATGAAG 1980

AACATTA 1987

Figure 3-1

Figure 3-2. Transmembrane prediction of the amino acid sequence of PeaD2R (TMHMM Server, V 2.0. http://www.cbs.dtu.dk/services/TMHMM/). The protein is predicted to contain seven transmembrane domains, which is a defining characteristic of G-protein coupled receptors. A large third intracellular loop is also predicted, which is commonly found in dopamine receptors.

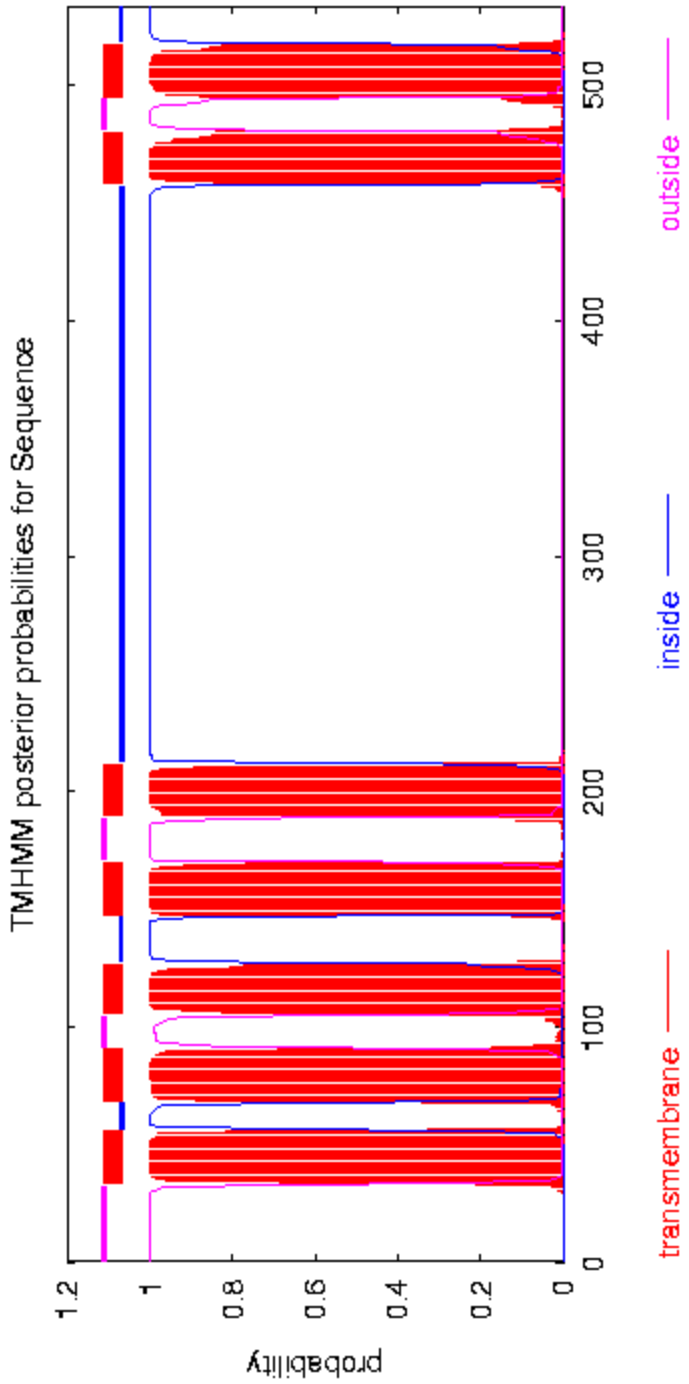
TMHMM posterior probabilities for Sequence
probability
1.2
1
0.8
0.6
0.4
0.2
0
0
100
200
300
400
500
transmembrane
inside
outside

Figure 3-2

Figure 3-3. Amino acid alignment of the *Periplaneta americana* D2-like dopamine receptor (PeaD2R) with D2-like receptors from other animals, including flour beetle *Triboleum castaneum* (TribolD2R), fruit fly *Drosophila melanogaster* (DrmD2R-PD), honeybee *Apis mellifera* (ApisD2R), and human *Homo sapiens* (HumanD2R). There are many regions of homology among the insect dopamine receptors, despite the diversity of insect orders. There is less homology between the insect and human receptors, but small homologous regions are present.

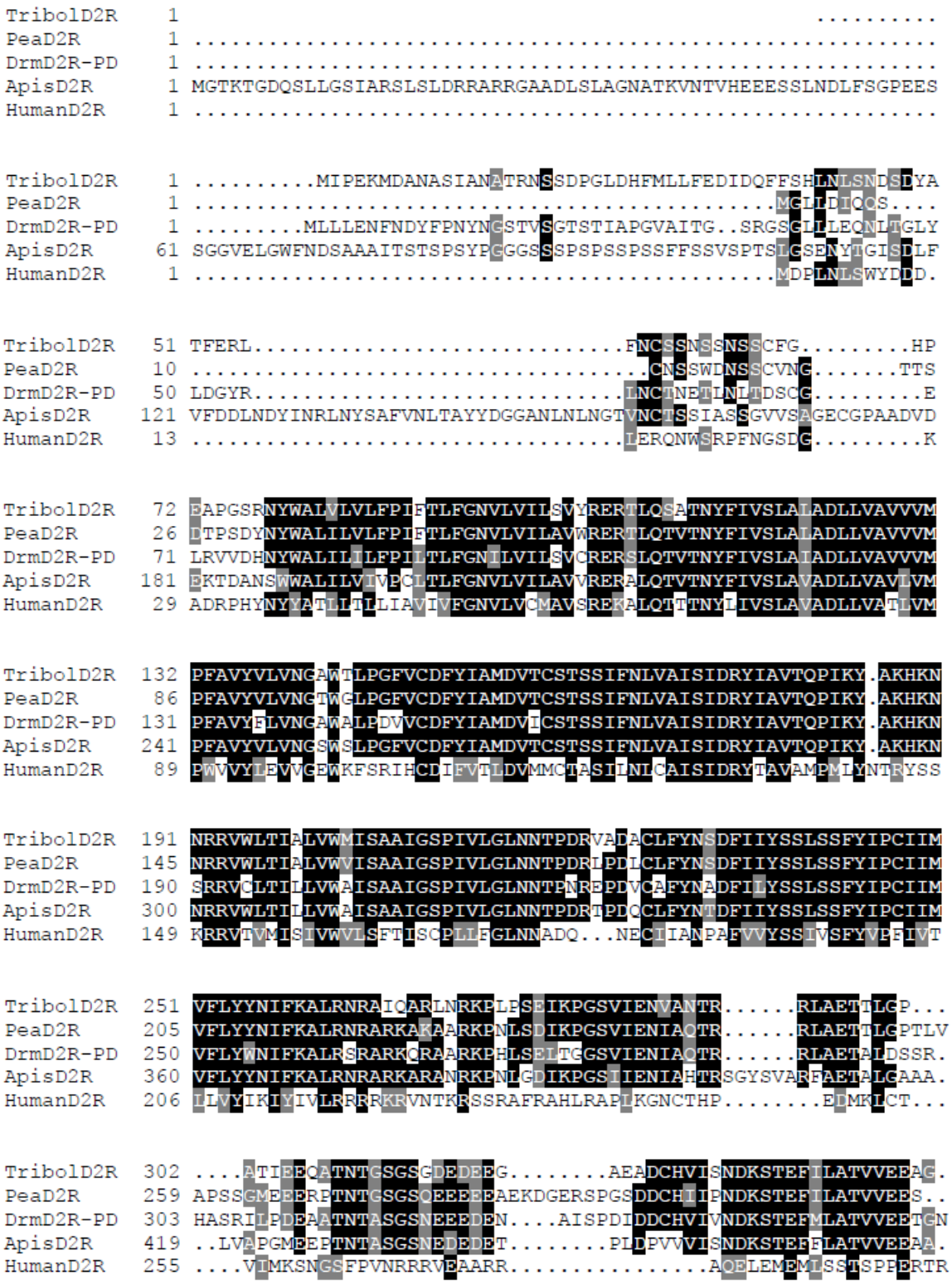

Figure 3-3

Figure 3-3, continued

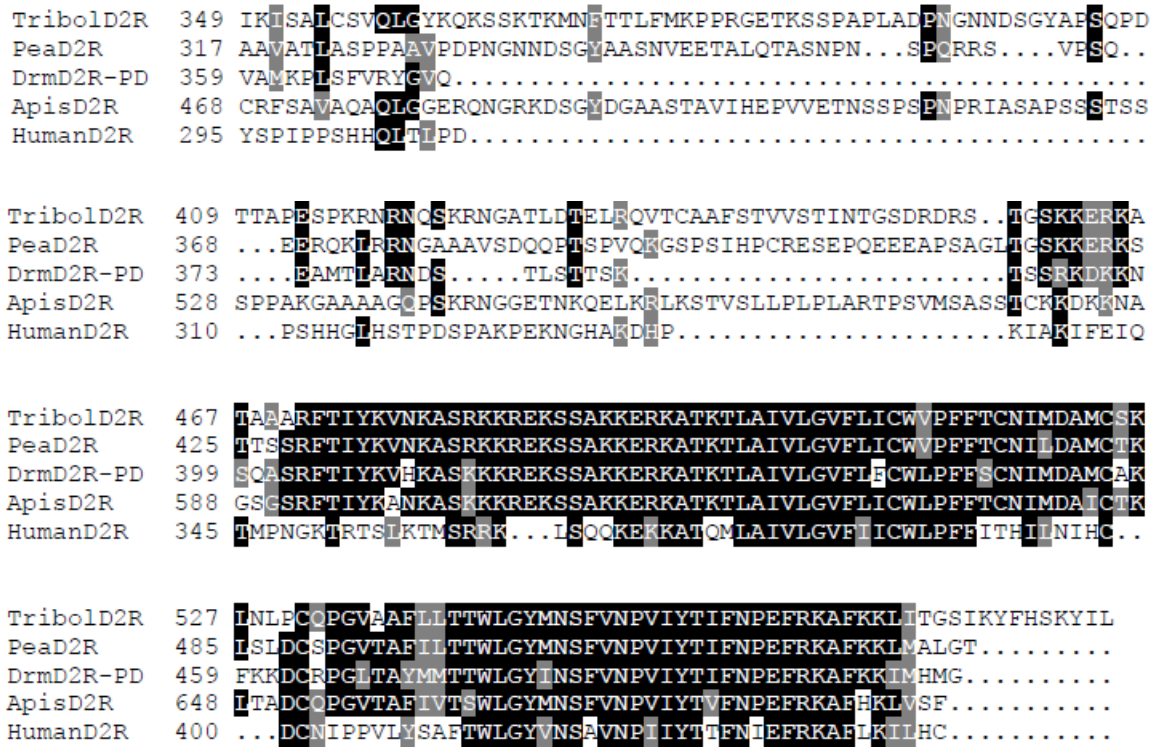

Figure 3-4. Phylogenetic analysis of dopamine receptors from various insect species. Dopamine receptor amino acid sequences from *Drosophila melanogaster* (Drm), *Apis mellifera* (Apm), *Anopheles gambiae* (Ang), *Nasonia vitripennis* (Nav), *Triboleum castaneum* (Trc), *Bombyx mori* (Bom), *Ctenocephalides felis* (Ctf), and *Acyrthosiphon pisum* (Acp) were analyzed. Three subtypes of insect dopamine receptors were analyzed: Dopr1 receptors (D1-like receptors analogous to the *Drosophila* dDA1 receptor), Dopr2 receptors (D1-like receptors analogous to the *Drosophila* DAMB receptor), and D2R (D2-like receptors analogous to *Drosophila* D2R). The three receptor subtypes were grouped into three distinct families, with the Dopr1 and Dopr2 receptors being more closely related to each other than to the D2-like receptors. The *Periplaneta americana* D2-like receptor (PeaD2R) was grouped with other insect D2-like receptors, indicating that it is truly a D2-like receptor, and evolutionarily distinct from the D1-like receptor subtypes.

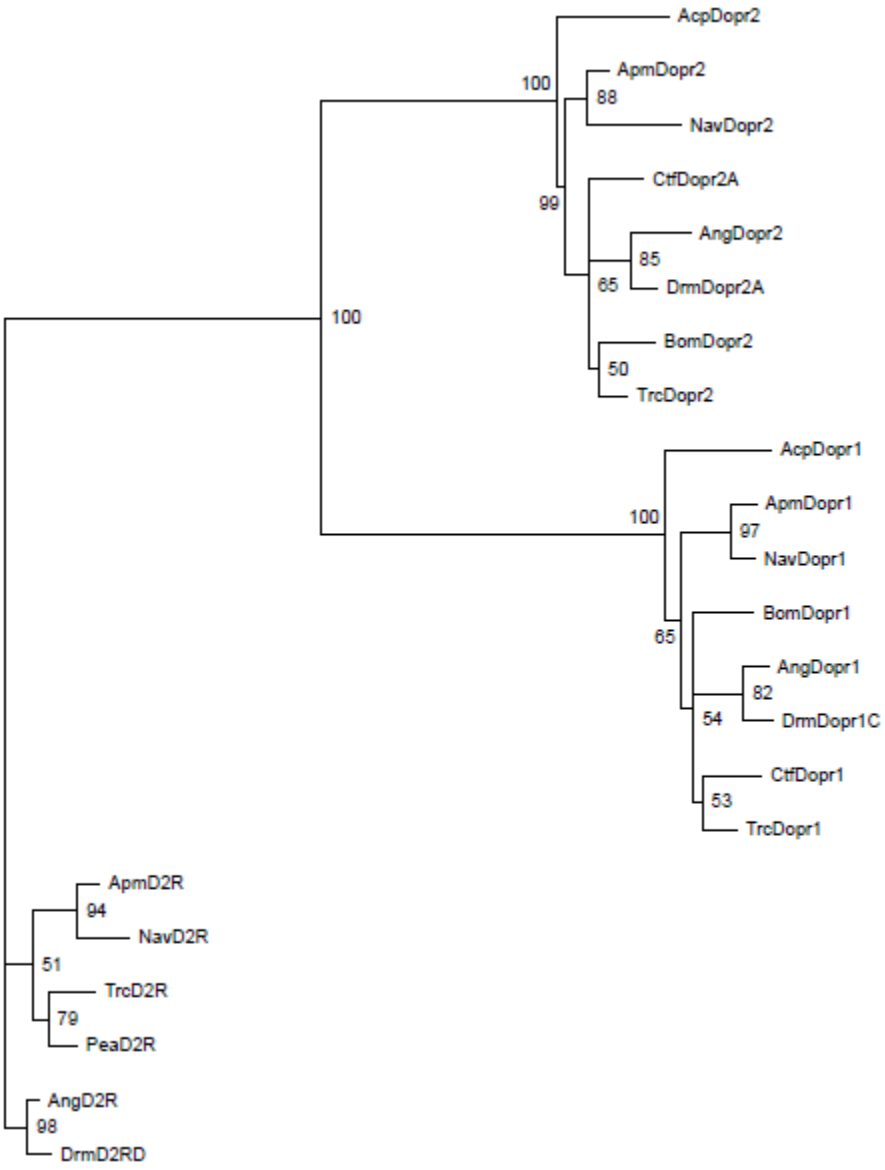
AcpDopr2
ApmDopr2
NavDopr2
CtfDopr2A
AngDopr2
DrmDopr2A
BomDopr2
TrcDopr2
AcpDopr1
ApmDopr1
NavDopr1
BomDopr1
AngDopr1
DrmDopr1C
CtfDopr1
TrcDopr1
ApmD2R
NavD2R
TrcD2R
PeaD2R
AngD2R
DrmD2RD
100
88
99
85
65
100
50
100
97
65
82
54
53
94
51
79
98

Figure 3-4

Figure 3-5. Aequorin-based luminescence assay comparing the efficiencies of three different chimeric G-proteins. Gqi5-HA, Gqo5-HA, and Gqs5-HA, along with apoaequorin and *Drosophila* D2R, were transfected into CHO-K1 cells. Each population of cells was treated with 5 μM dopamine, and the corresponding luminescence response was measured. The luminescence response in relative luminescence units was strongest in cells expressing Gqo5-HA, compared to Gqi5-HA and Gqs5-HA. This implies that this G-protein coupled receptor is preferentially coupled to Gαo, and this chimera was used for all subsequent experiments with insect D2-like receptors.

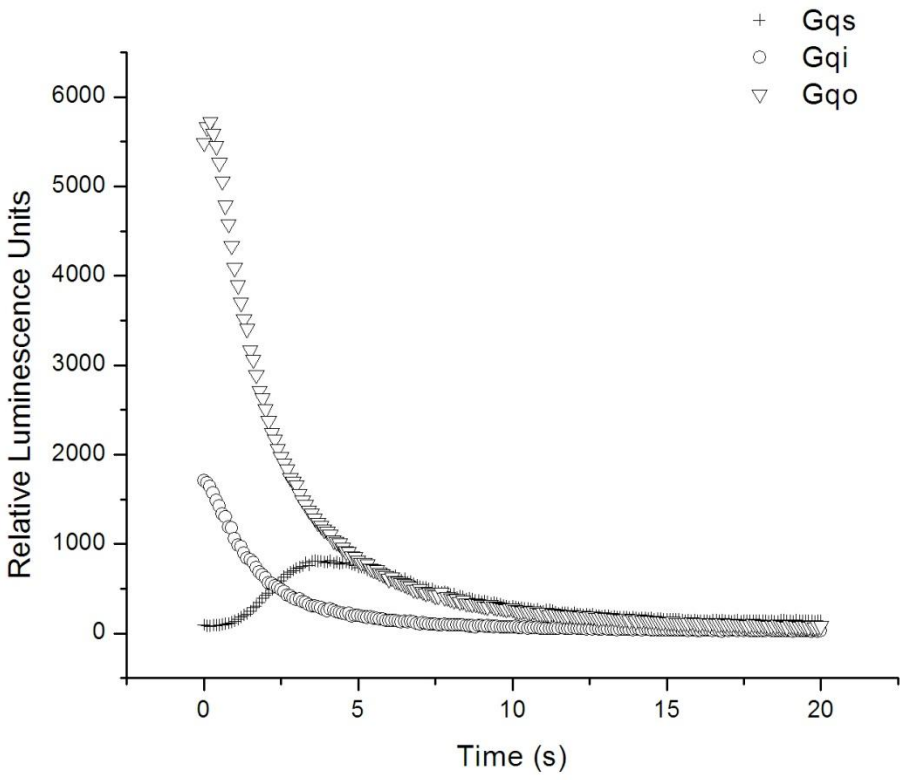
Gqs
Gqi
Gqo
Relative Luminescence Units
6000
5000
4000
3000
2000
1000
0
0
5
10
15
20
Time (s)

Figure 3-5

Figure 3-6. Luminescence responses in relative luminescence units (RLU) of the D2-like dopamine receptor from *Periplaneta americana* (PeaD2R) to varying concentrations of dopamine. There is a direct correlation between dopamine concentration and luminescence response, which permits the examination of ligand-receptor dose response relationships. Plotting these data as a function of concentration forms a sigmoidal curve that is described by the logistic equation.

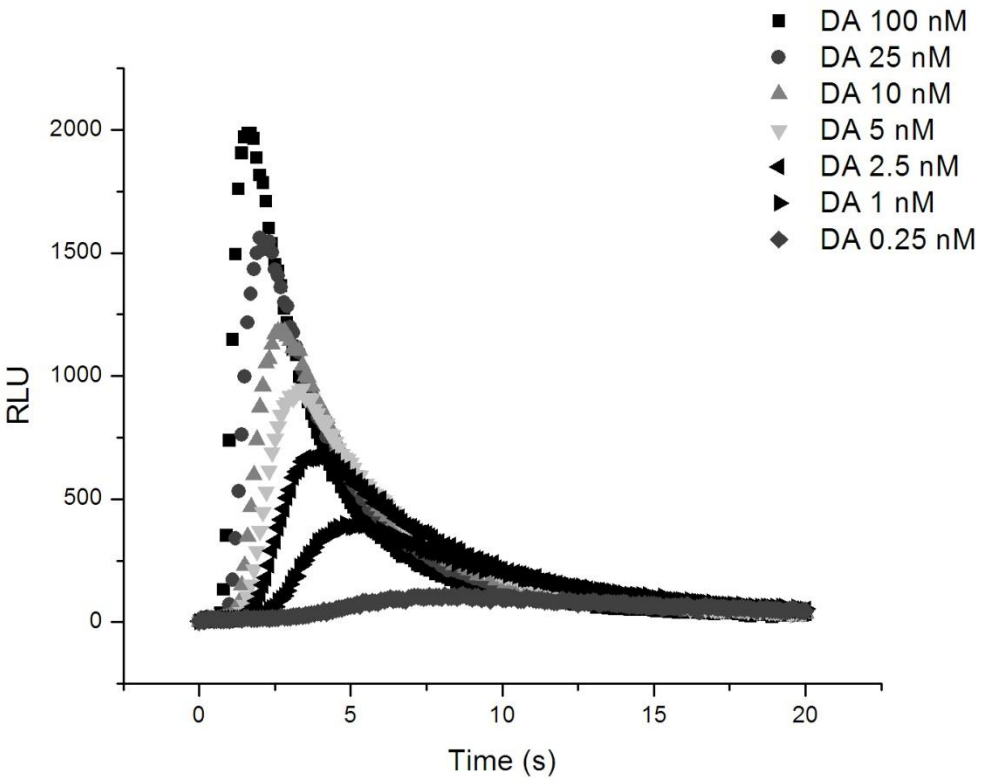
DA 100 nM
DA 25 nM
DA 10 nM
DA 5 nM
DA 2.5 nM
DA 1 nM
DA 0.25 nM
RLU
2000
1500
1000
500
0
0
5
10
15
20
Time (s)

Figure 3-6

Figure 3-7. Dose response curves of dopamine, norepinephrine, serotonin, tyramine, and octopamine for PeaD2R. Each reading was done in quadruplicate, and the total luminescence was averaged over the four readings, and expressed as % relative luminescence units (RLU) normalized to the maximum response. Dopamine was the most potent ligand, with an EC_{50} of 1.39 nM. The EC_{50}s of norepinephrine, serotonin, tyramine, and octopamine were 206 nM, 928 nM, 1140 nM and 38300 nM respectively. Error bars represent standard deviations from the mean.

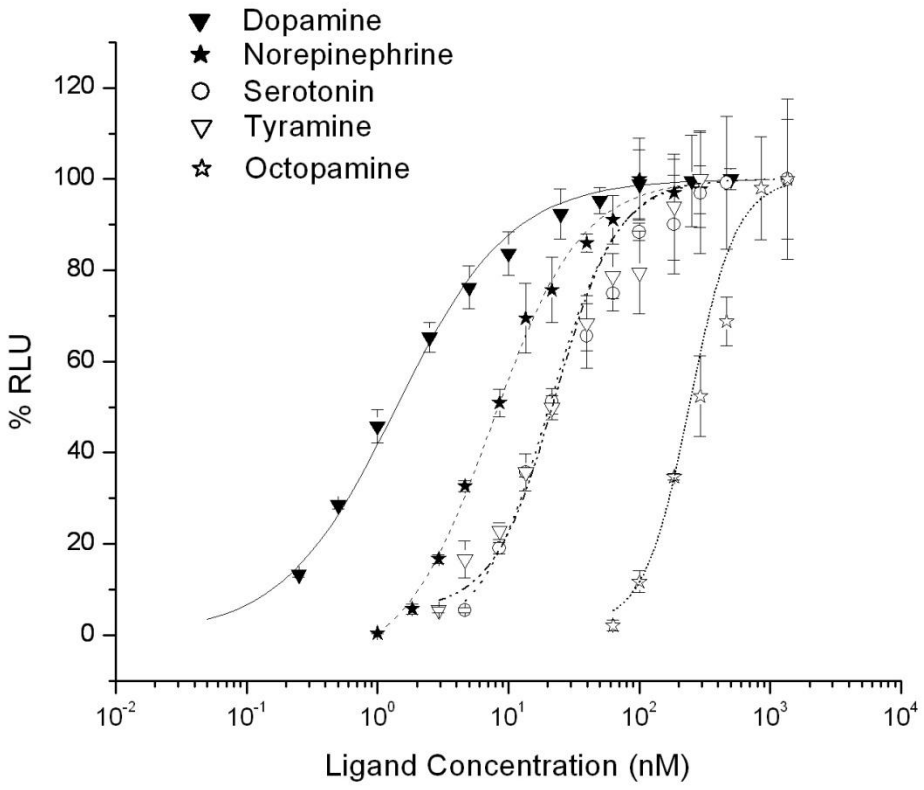
Dopamine
Norepinephrine
Serotonin
Tyramine
Octopamine
% RLU
120
100
80
60
40
20
0
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
Ligand Concentration (nM)

Figure 3-7

Figure 3-8. Dose response curves for synthetic agonists for PeaD2R. The nonselective agonist 6,7-ADTN was the best synthetic ligand, with an EC_{50} of 6.12 nM. The other nonselective agonist apomorphine was also an effective ligand, with an EC_{50} of 101 nM. The D2-selective ligands bromocriptine and quinpirole had EC_{50} values of 153 nM and 21600 nM respectively. The mammalian D1-selective ligands SKF 82958 and SKF 38393 also had considerable activity at the receptor, with EC_{50}s of 621 nM and 1818 nM respectively. Error bars represent standard deviations from the mean.

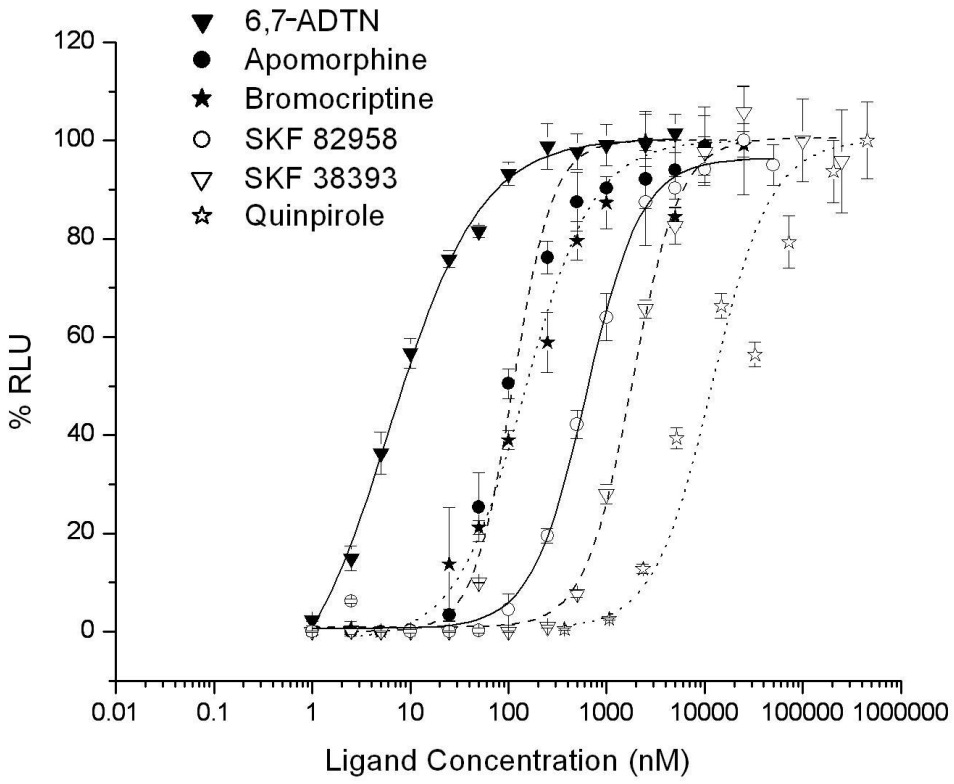
6,7-ADTN
Apomorphine
Bromocriptine
SKF 82958
SKF 38393
Quinpirole
% RLU
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-8

Figure 3-9. Inhibition curves of synthetic antagonists for PeaD2R. Antagonists competed against dopamine (5 nM) for receptor binding. The mammalian D2-selective compound sulpiride and the mammalian D1-selective compound SCH 23390 have nearly identical inhibition curves (IC_{50}s of 1410 nM and 1340 nM respectively). The mammalian D2-selective antagonist fallypride, and the nonselective antagonist flupenthixol were less effective, but still blocked receptor activation by dopamine with IC_{50}s of 9930 nM and 28500 nM respectively. Error bars represent standard deviations from the mean.

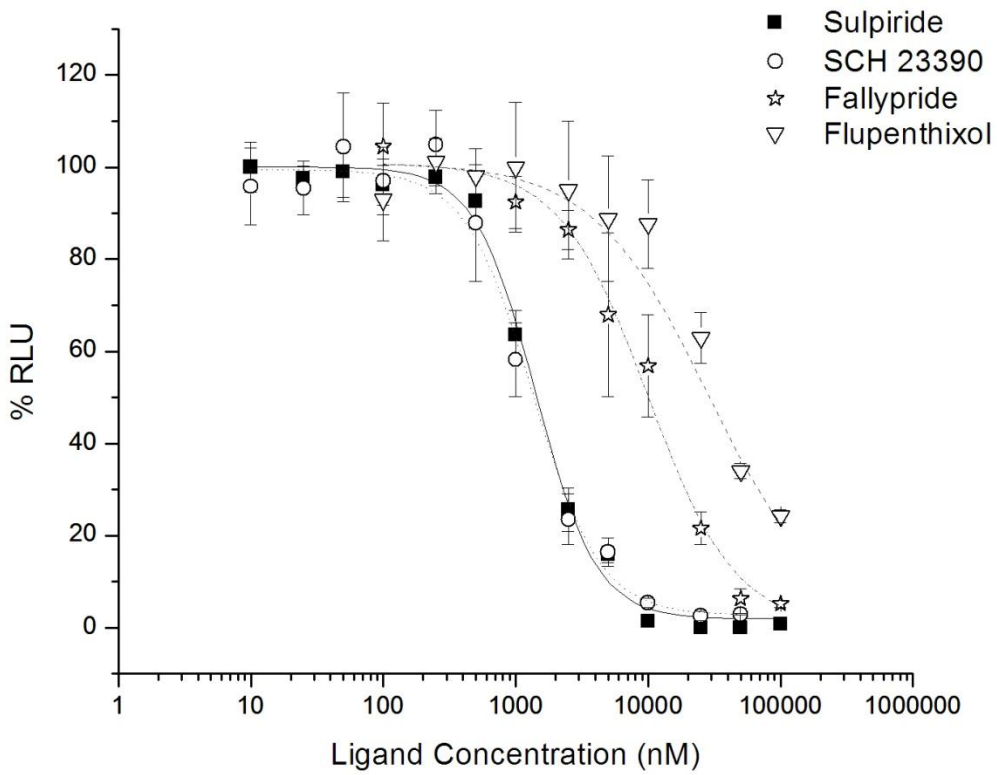
Sulpiride
SCH 23390
Fallypride
Flupenthixol
% RLU
120
100
80
60
40
20
0
1
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-9

Figure 3-10. Dose response curves of biogenic amines for one of the splice variants of the *Drosophila* D2-like receptor, D2R-589. Dopamine was the most potent ligand, with an EC_{50} of 21.9 nM. The EC_{50}s for norepinephrine, serotonin, tyramine, and octopamine were 893 nM, 2490 nM, 7520 nM**,** and 136000 nM respectively. These values were approximately 2-10 times larger than corresponding values for the cockroach D2-like receptor, but the ascending order of potency was unchanged. Error bars represent standard deviations from the mean.

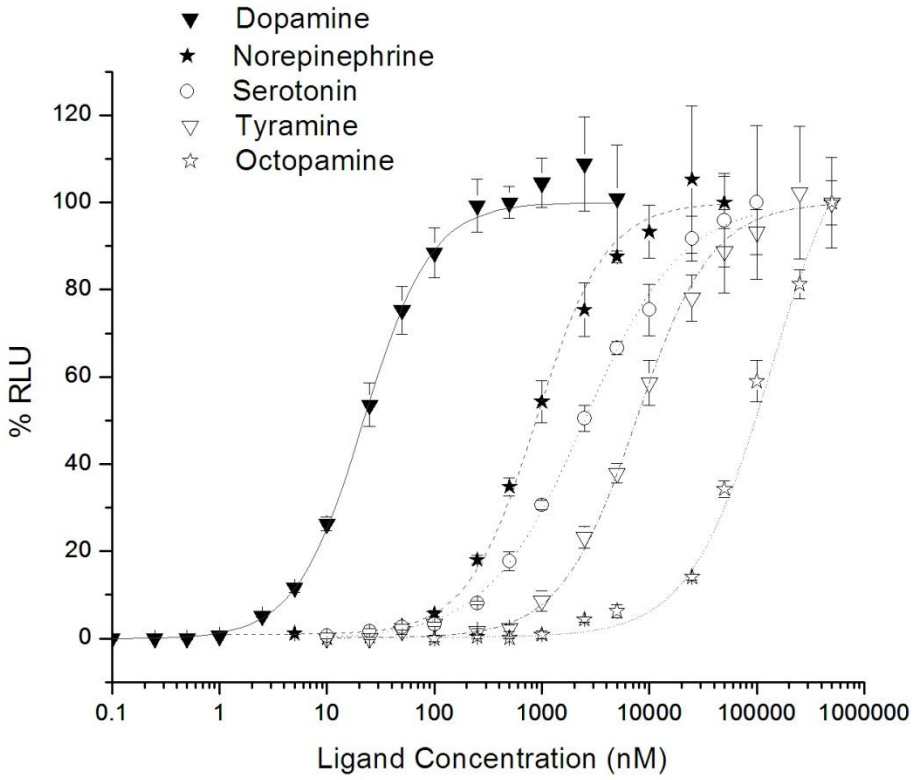
Dopamine
Norepinephrine
Serotonin
Tyramine
Octopamine
% RLU
120
100
80
60
40
20
0
0.1
1
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-10

Figure 3-11. Dose response curves of synthetic agonists for *Drosophila* D2R-589. 6,7-ADTN and apomorphine were the best agonists, with EC_{50}s of 62.6 nM and 85.5 nM respectively. Bromocriptine was the best D2-selective compound, with an EC_{50} of 612 nM. SKF 82598, SKF 38393, and quinpirole also triggered receptor activity, with EC_{50}s of 4700 nM, 14200 nM, and 165000 nM respectively. The agonist EC_{50} values of this receptor were larger compared to the corresponding values of the cockroach D2-like receptor, but the order of potency remained unchanged. This is consistent with the biogenic amine dose-response data for the two receptors. Error bars represent standard deviations from the mean.

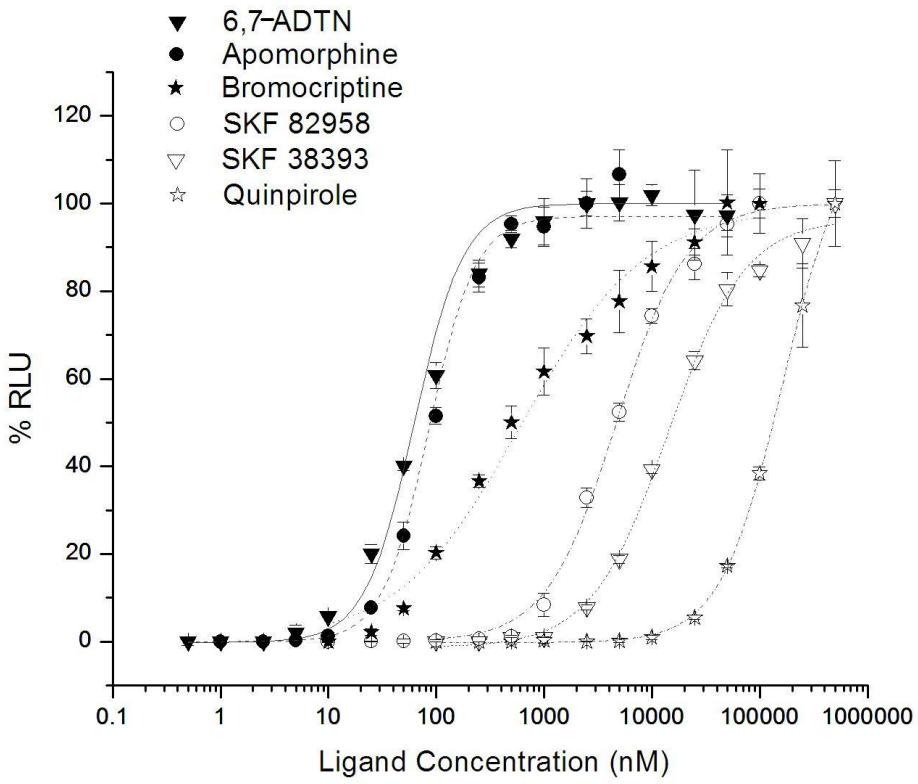
6,7-ADTN
Apomorphine
Bromocriptine
SKF 82958
SKF 38393
Quinpirole
% RLU
120
100
80
60
40
20
0
0.1
1
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-11

Figure 3-12. Inhibition curves of synthetic antagonists for *Drosophila* D2R-589. Antagonists competed against dopamine (50 nM) for receptor binding. Similar to PeaD2R, sulpiride and SCH 23390 were the most effective antagonists tested, with IC_{50}s of 21000 nM and 21600 nM respectively. However, flupenthixol was a better antagonist (IC_{50} = 49300 nM) than fallypride (IC_{50} = 247000 nM). Error bars represent standard deviations from the mean.

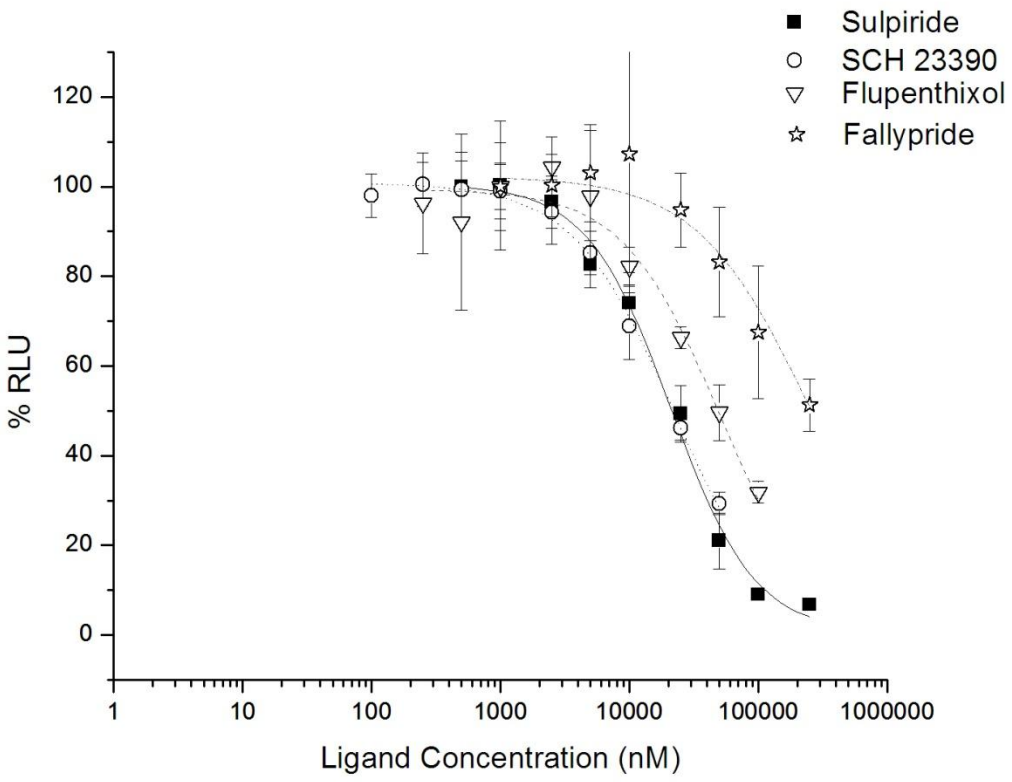
Sulpiride
SCH 23390
Flupenthixol
Fallypride
% RLU
120
100
80
60
40
20
0
1
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-12

Figure 3-13. *Drosophila* DAMB agonist dose-response curves. Dopamine was the best endogenous ligand, with an EC_{50} of 2.72 nM. 6,7-ADTN was also very potent, with an EC_{50} of 2.83 nM. The nonselective agonist apomorphine induced receptor activity with an EC_{50} of 618 nM, whereas the D1-selective agonists SKF 82958 and SKF 38393 had EC50s of 1810 nM and 4476 nM respectively. Surprisingly, the mammalian D2-selective compound quinpirole was effective at DAMB, with an EC_{50} of 6480 nM. Error bars represent standard deviations from the mean.

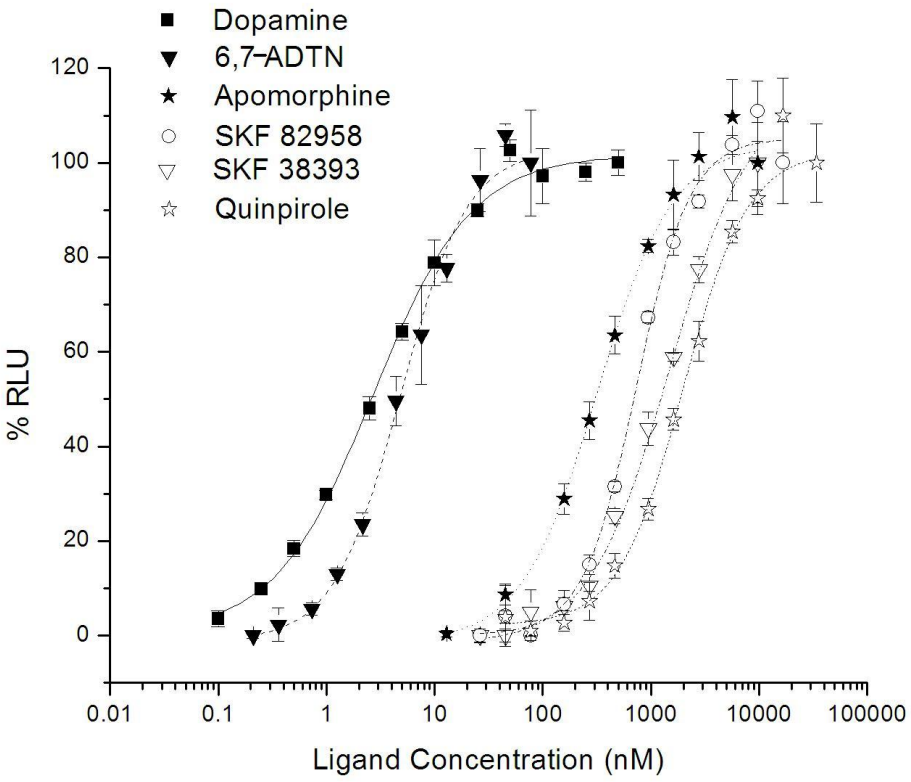
Dopamine
6,7-ADTN
Apomorphine
SKF 82958
SKF 38393
Quinpirole
% RLU
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
100000
Ligand Concentration (nM)

Figure 3-13

Figure 3-14. Inhibition curves for *Drosophila* DAMB receptor. SCH 23390 was the only antagonist tested that was able to fully suppress dopamine-induced luminescence, with an IC_{50} of 3309 nM. Flupenthixol and mianserin only partially suppressed the luminescence response, to 36.1% and 46% of the maximum dopamine-induced luminescence respectively. Error bars represent standard deviations from the mean.

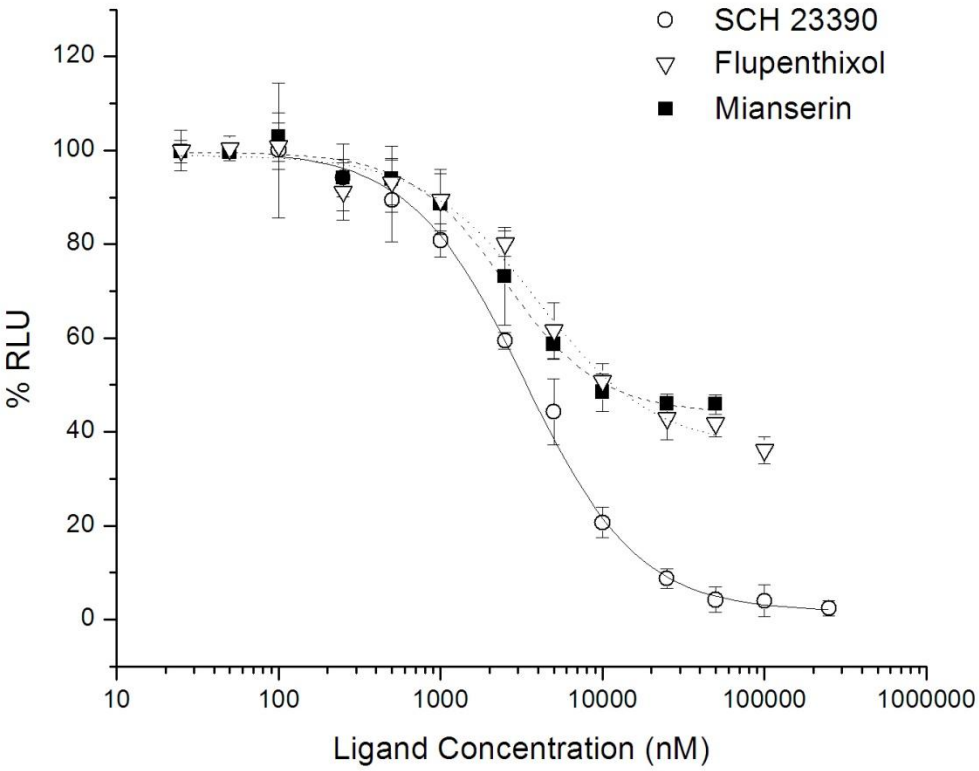
SCH 23390
Flupenthixol
Mianserin
% RLU
120
100
80
60
40
20
0
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-14

Figure 3-15. Dopamine-induced luminescence upon dDA1 activation in two different cell types. *Drosophila* dDA1 receptor was expressed in both mammalian Chinese hamster ovary cells (CHO-K1) and insect Hi5 cells (*Trichoplusia ni*). Dopamine was 10 times more potent in Hi5 cells expressing dDA1 (EC_{50} = 652 nM) compared to CHO-K1 cells (EC_{50} = 6728 nM). Error bars represent standard deviations from the mean.

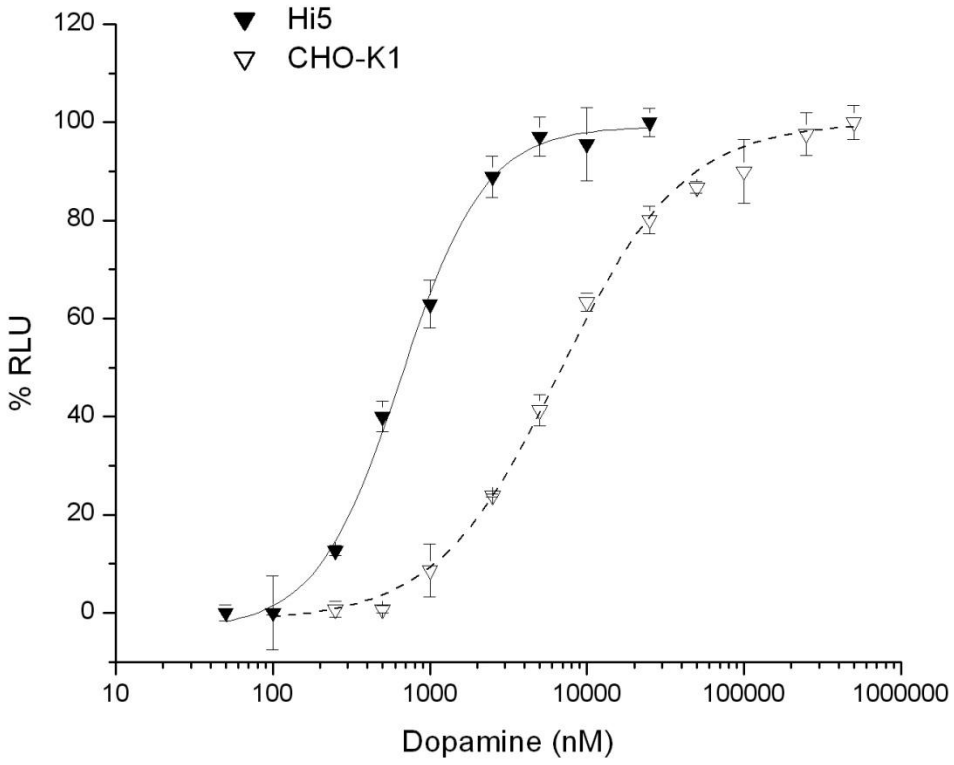
Hi5
CHO-K1
% RLU
120
100
80
60
40
20
0
10
100
1000
10000
100000
1000000
Dopamine (nM)

Figure 3-15

Figure 3-16. Agonist dose response curves for dDA1 expressed in Hi5 cells. 6,7-ADTN was the only synthetic ligand tested that demonstrated activity at the receptor (EC_{50} = 2751 nM). Error bars represent standard deviations from the mean.

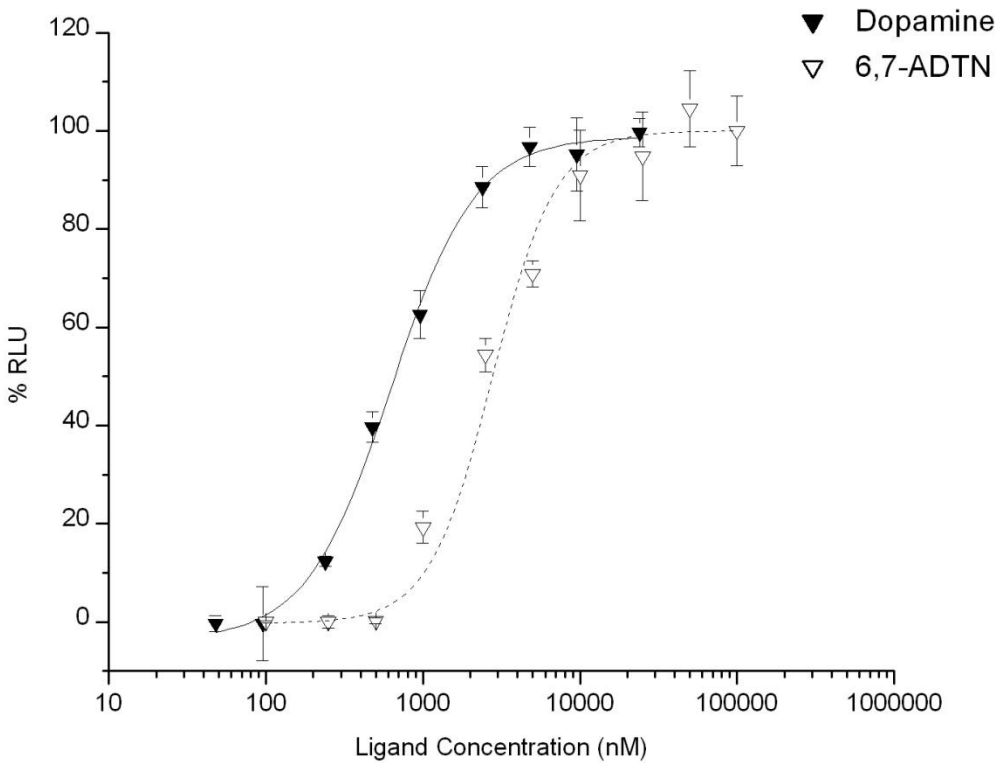
Dopamine
6,7-ADTN
% RLU
120
100
80
60
40
20
0
10
100
1000
10000
100000
1000000
Ligand Concentration (nM)

Figure 3-16

Figure 3-17. Inhibition curves for *Drosophila* dDA1 receptor. SCH 23390 and mianserin were able to fully suppress dopamine-induced luminescence, with IC_{50}s of 424 nM and 1410 nM respectively. Flupenthixol and butaclamol also suppressed dopamine-induced luminescence (IC_{50}s of 4470 nM and 13900 nM respectively), but the inhibition curves appear to plateau at 85-90% of the control values, suggesting noncompetitive inhibition. Error bars represent standard deviations from the mean.

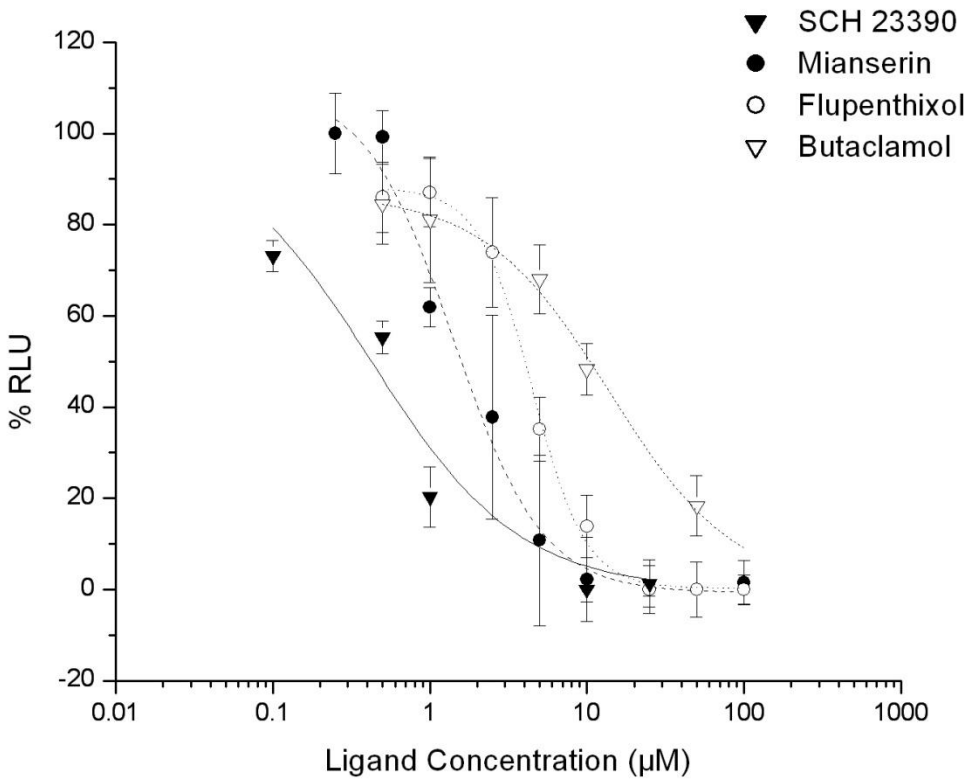
SCH 23390
Mianserin
Flupenthixol
Butaclamol
% RLU
120
100
80
60
40
20
0
-20
0.01
0.1
1
10
100
1000
Ligand Concentration (μM)

Figure 3-17

Figure 3-18. Duration of cockroach grooming in response to being stung by *Ampulex compressa*. Ten µL of compound solution or buffer controls (0.1% acetic acid or phosphate buffered saline, PBS) were injected into the cockroach abdominal hemolymph, and animals were stung 1 hour later. Grooming behavior was measured for 1 hour after the initial onset of grooming. Sulpiride injection caused a slight but significant decrease in grooming time (27.7 min ± 4 min, n = 10, p = .038) compared to control injection (32.5 min ± 5.4 min, n = 10). Error bars represent standard deviations from the mean. Neither bromocriptine (10 nmoles, n=10), SCH 23390 (1 nmole, n=10), nor flupenthixol (1 nmole, n=9) pretreatment produced significantly different grooming times from their respective controls. Two sample t-tests were performed to determine statistical significance.

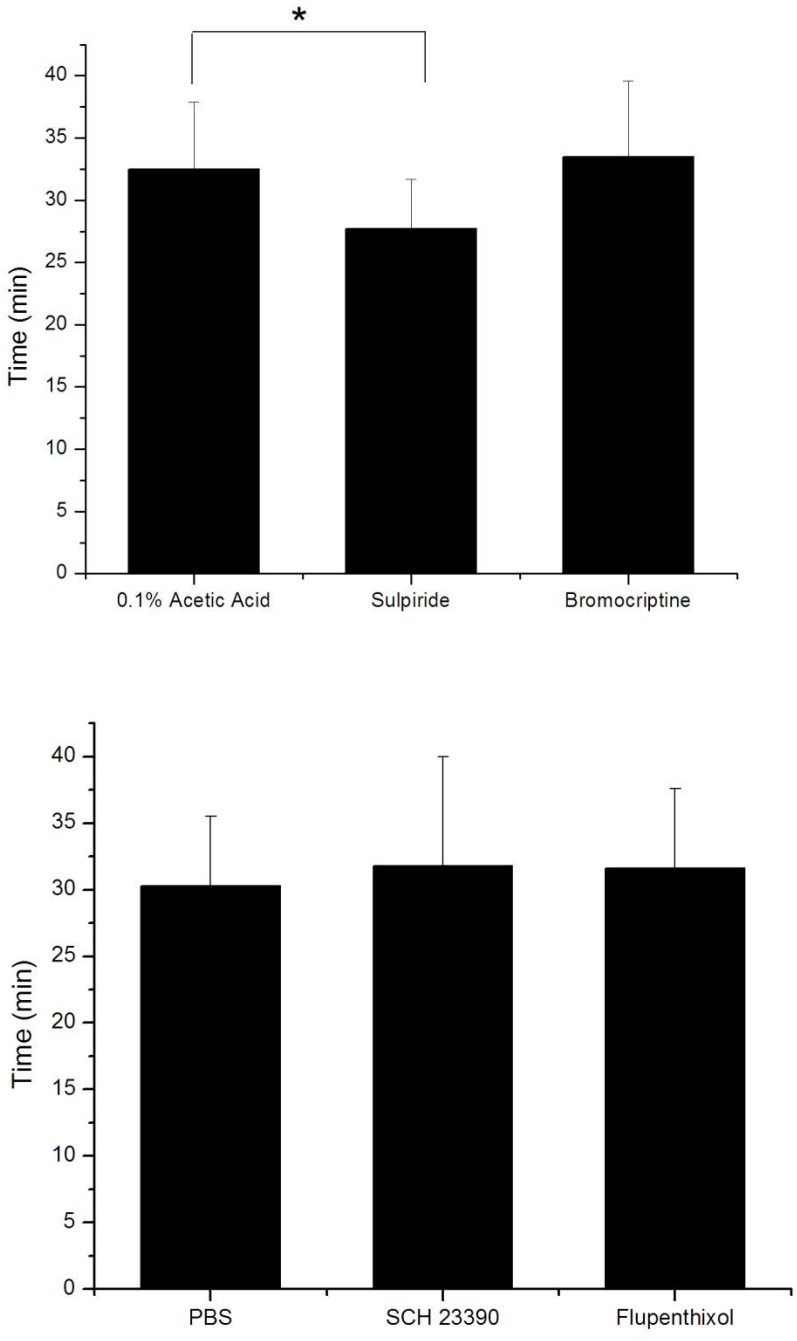
*
Time (min)
0
5
10
15
20
25
30
35
40
0.1% Acetic Acid
Sulpiride
Bromocriptine
Time (min)
0
5
10
15
20
25
30
35
40
PBS
SCH 23390
Flupenthixol

Figure 3-18

Chapter Four

A Functional Analysis of Venom Components from the Parasitoid Wasp *Ampulex compressa*

Abstract

The parasitoid wasp *Ampulex compressa* injects venom directly into the brain and subesophageal ganglion of the cockroach *Periplaneta americana* to induce long-lasting hypokinesia. The venom is a cocktail of proteins, peptide, and compounds of low molecular weight that acts upon the central nervous system to suppress the generation of spontaneous movements and escape responses in cockroaches. In this study, I attempted to determine biological functions of the venom and two venom peptides, DG2807 and DG2847, and relate function to hypokinesia induction. A battery of investigative assays was performed to determine functional properties of milked venom and venom peptides, examining antimicrobial properties, lytic effects on cells *in vitro*, behavioral effects, and effects on dopamine receptor activity. Neither milked venom nor venom peptides prevented the growth of *Escherichia coli* or *Bacillus thuringiensis* on agar plates. Exposure to DG2847, DG2807, or milked venom did not induce cell death in Chinese hamster ovary cells (CHO-K1) or Hi5 cells (*Trichoplusia ni*). Injection of DG2807, but not DG2847, into the central complex of the brain reduced spontaneous locomotion in cockroaches, suggesting that this peptide is directly involved in hypokinesia induction. Using an aequorin-based luminescence assay, the activity of D2-like receptors (PeaD2R from *Periplaneta americana*) expressed in cultured cells was assessed in the presence of venom or venom peptides. Coapplication of DG2807 and DG2847 with dopamine did not suppress receptor activation, but incubation for >30 minutes reduced both dopamine-induced and ATP-induced luminescence in a dose-dependent manner. Because ATP acts at a different receptor than dopamine, this suggests that the peptides do not specifically

target dopamine receptors, but act on a common downstream target for both receptors. These peptides may prevent intracellular calcium mobilization, or reduce the impermeability of the cell membrane to calcium through pore/channel formation.

Introduction

Animal venoms are complex mixtures of bioactive molecules, including proteins, peptides, and compounds of low molecular weight, such as polyamines and biogenic amines (Abe *et al.* 1989; Blagbrough *et al.* 1994; Hisada *et al.* 2005; Moreau *et al.* 2005). Many venom toxins have evolved to affect specific biological targets, which make them good pharmacological tools to study cellular processes. Venoms are also rich sources of novel bioactive components, which may serve as the basis for new drug discovery.

The solitary wasp *Ampulex compressa* (Hymenoptera: Ampulicidae) uses its venom to subdue the host of its larva, the American cockroach *Periplaneta americana*. The wasp initially stings directly into the first thoracic ganglion, which causes a transient paralysis of the prothoracic legs for approximately three minutes. The wasp executes a second, very precise sting directly into the brain and subesophageal ganglion (SEG) which induces a bout of grooming in the cockroach lasting approximately 30 minutes. The cockroach subsequently falls into a state of hypokinesia, characterized by suppression of escape responses and an elevated threshold for walking initiation (Gal *et al.* 2008). The wasp manipulates the hypokinesic cockroach, leading it to a burrow whereupon it oviposits on the coxa and subsequently buries the cockroach with debris.

Larvae hatch a few days later, and feed on living cockroach tissue until pupation. An adult wasp emerges from the dead cockroach approximately six weeks after oviposition (Libersat 2003; Gal *et al.* 2005).

The sting into the head cavity induces both stereotypic grooming behavior and long-term hypokinesia. The grooming behavior invariably follows the head sting, and does not occur if the wasp is removed from the cockroach before the second sting (Weisel-Eichler *et al.* 1999). This indicates that grooming is induced by venom injection and not mechanical irritation from the stinging process. Dopamine, or a dopamine-like substance in the venom, is implicated in the induction of grooming behavior. First, preliminary experiments identified the presence of a venom component with the exact same molecular weight as dopamine (Weisel-Eichler *et al.* 1999). Secondly, grooming was elicited in naïve cockroaches by injecting the hemolymph or SEG with dopamine and dopamine agonists (Weisel-Eichler *et al.* 1999). In addition, pretreating cockroaches with the dopamine receptor antagonist sulpiride led to reductions in sting-induced grooming duration (Chapter 3). It is unknown whether grooming is an intended response to venom injection, or simply an unintended consequence of hypokinesia. It is possible that compulsive grooming prevents the cockroach from moving away while the wasp prepares a burrow (Weisel-Eichler *et al.* 1999). The wasp may also induce grooming in order to ensure that the areas where oviposition will occur are free from contaminants or bacteria that could affect the survival of the wasp larva.

Ampulex venom is a cocktail of proteins, peptides, and compounds of low molecular weight. Currently, only a small number of venom components have been

identified. GABA, β-alanine, and taurine (9-25 mM) in the venom are responsible for the transient paralysis of the front legs by increasing GABA-mediated chloride ion conductance, leading to hyperpolarization of central neurons and a block of action potential production (Moore *et al.* 2006). Previous evidence suggests that dopamine is present in the venom, and responsible for grooming induction (Weisel-Eichler *et al.* 1999). In addition, full or partial nucleotide and amino acid sequences of four venom peptides (DG2807, DG2847, DG5842, and DG7577) have been determined (Moore 2003), but their specific functions are unknown.

An unidentified peptidergic venom component forms monovalent cation channels in lipid bilayers. The precise function of this venom-induced channel has not been determined, but it may facilitate the movement of other venom components into cells or possess antibacterial properties (Gincel *et al.* 2004). Some pore-forming peptides display antimicrobial activity (Christensen *et al.* 1988; Mysliwy *et al.* 2009), and many antimicrobial peptides have been identified in hymenopteran species, including *Polistes dominulus* (Turillazzi *et al.* 2006), *Anoplius samariensis* (Konno *et al.* 2001), *Vespa bicolor* Fabricius (Chen *et al.* 2008), and *Vespa magnifica* (Xu *et al.* 2006).

The mechanism underlying hypokinesia is currently unknown, but evidence implicates dopamine receptor dysfunction as a possible cause. Hypokinesia is phenotypically similar to Parkinson's disease, whose symptoms include bradykinesia and the inability to generate spontaneous movements. Parkinson's disease is characterized by a deficiency in dopaminergic signaling, due to the degeneration of dopaminergic neurons in the substantia nigra. Previous data show that biogenic amine levels are not depressed

in stung animals (Chapter 2), indicating that dopamine synthesis and storage are unaffected by *Ampulex* venom. Treating cockroaches with the dopamine receptor antagonist flupenthixol reduces the cockroach escape response (Weisel-Eichler *et al.* 2002). In addition, injection of dopamine into naïve cockroaches induces grooming behavior, whereas dopamine injection into stung animals does not. This implies that envenomation by *Ampulex compressa* renders the cockroach insensitive to the effects of dopamine. The restoration of dopamine-induced grooming in stung animals follows the same time course for recovery from hypokinesia (Weisel-Eichler *et al.* 2002), suggesting that the two phenomena share a similar mechanism of action.

It is also possible that hypokinesia induction is elicited through venom-induced neuronal death. The wasp's stinger directly pierces the head ganglia, injecting venom into the central complex and mushroom bodies of the brain, and the midline of the SEG (Haspel *et al.* 2003). These areas are very important for locomotor activity (Martin *et al.* 1999; Strausfeld 1999; Gal *et al.* 2006), so it is conceivable that venom-induced damage to neurons in these areas may lead directly to locomotor impairment. However, because hypokinesia is reversible, neuronal regeneration, which has been observed in insects (Patschke *et al.* 2004), would need to occur for the cockroach to fully recover from envenomation.

In this study, we investigated chemical and functional properties of *Ampulex* venom, attempting to link specific venom peptides to hypokinesia induction and compulsive grooming following the head sting. We tested milked venom and synthetic venom peptides DG2807 and DG2847 for antimicrobial activity, and whether direct

application of these compounds induces cell death. These two venom peptides were also injected into the central complex of the cockroach brain to investigate their potential functions *in vivo*, and whether either of these peptides induces symptoms of hypokinesia. We analyzed the venom for dopamine and octopamine using high performance liquid chromatography with electrochemical detection (HPLC-ED). We also used an aequorin-based luminescence assay to investigate venom and venom peptide effects on receptor-induced calcium elevation *in vitro*. Activation of the D2-like receptor from *Periplaneta americana* and an endogenous purinergic receptor was examined in the presence of venom peptides and milked venom to determine if the venom selectively targets dopamine receptors, or interferes with signaling pathways common to both receptors.

Materials and Methods

Animal Maintenance. *Ampulex compressa* were maintained in a rearing room at 27° C in 16 in x 16 in x 24 in plexiglass cages with moderate humidity. They were fed honey and water separately *ad libitum*, and kept under a 16:8 light-dark cycle. *Periplaneta americana* were raised in garbage cans with eggshell cartons on dry dog food (Valutime) and water *ad libitum*.

Venom Extraction. To extract venom from live animals, wasps were anesthetized with carbon dioxide, and placed in the end of a modified 1000 µL pipet tip with the abdomen extending outward. The wasp was allowed to sting through a piece of parafilm, and each venom droplet produced was collected in deionized water, and kept on dry ice to preserve biological activity. Venom was stored at -20° C until immediately before use.

HPLC-ED Analysis of Venom. HPLC-ED was performed to confirm the presence of dopamine in *Ampulex* venom, and potentially identify other low molecular weight compounds. Seventy-three sting equivalents in 20 µL were loaded onto an ESA model 580 isocratic solvent delivery module (ESA, Chelmsford, MA) connected to a reversed-phase HPLC column and an ESA Coulochem II electrochemical detector. Separation was performed on a 250 x 4.6 mm, 5 µm particle size, 120 Å pore size Clipeus C18 reversed-phase column (Higgins Analytical, Mountain View, CA) with a flow rate of 1.0 mL/min. The mobile phase consisted of 50 mM sodium phosphate buffer with 5 mM heptanesulfonic acid, 10% acetonitrile, adjusted to a pH of 4.5 with phosphoric acid. A series of two porous graphite electrodes, a screening electrode and oxidizing electrode (at potentials of 100 mV and 650 mV respectively), were used to identify electroactive compounds in the samples, and generate peaks on the chromatogram. A standard mix of 11 chemicals (L-3,4-dihydroxyphenylalanine, tyrosine, 6-hydroxydopamine, 3,4-dihydroxyphenylacetic acid, norepinephrine, octopamine, tryptophan, homovanillic acid, dopamine, tyramine, and serotonin) was analyzed, and retention times were compared against unknown compounds in the venom. A standard curve for known quantities of chemicals in the standard mix (2, 1, and 0.5 pmoles) was generated. Data were quantified by integration of peak area, and compound levels in the venom sample were determined by interpolation of the standard curve. All compounds were purchased from Sigma Aldrich (St. Louis, MO).

Antimicrobial Effects. Wasp venom and synthetic venom peptides DG2807 and DG2847 (Moore 2003) were tested for antimicrobial activity against one gram-positive

bacterium (*Bacillus thuringiensis*) and one gram-negative bacterium (*Escherichia coli*, strain DH5α). Bacterial strains were grown in Luria broth, and were plated out onto a thin agar plate. Three µL of 200 µM DG2847 or DG2807 (or a combination of both) were applied to specific areas on the surface of the agar and allowed to dry. Seventy-one sting equivalents were spread in a specific area demarcated on each plate, and allowed to dry. Droplets of the antibiotic carbenicillin (50 mg/mL in water) were applied to a separate plate, and served as a positive control. Negative control areas (areas without the addition of venom/peptides) were also designated on each plate. Plates were incubated at 37º C overnight and checked the following day for growth. *Bacillus thuringiensis* was a gift from Dr. Sarjeet Gill, University of California, Riverside.

Cell Viability. Cell viability of Chinese hamster ovary (CHO-K1) cells and Hi5 cells (*Trichoplusia ni*) in the presence of venom and venom peptides was assessed. CHO-K1 cells were detached from their culture dish using a solution of phosphate buffered saline (PBS) and ethylenediaminetetraacetic acid (EDTA) and were resuspended in DMEM/F12 cell culture media (Invitrogen, Carlsbad, CA) supplemented with 1% bovine serum albumin (Sigma Aldrich) and 1% penicillin/streptomycin (Invitrogen) (referred to as BSA media). Cells were pelleted and washed in BSA media, and diluted to a concentration of 4-5 x 10^6 cells/mL in 1.5 mL tubes. Hi5 cells were detached by knocking the side of the culture flask, and pipetted into 1.5 mL tubes at a concentration of approximately 3-4 x 10^5 cells/mL. Cells were incubated with water, 200 µM DG2807, 200 µM DG2847, a 200 µM mixture of both venom peptides, or with milked venom (63 sting equivalents for CHO-K1 cells, 50 sting equivalents for Hi5 cells) in a 200 µL suspension. Treated cells

and control cells were incubated for 40 minutes at room temperature. To assess cell viability, trypan blue (0.4%) (Sigma Aldrich) was added (1:1) and after one minute, living and dead cells were counted on a hemocytometer. Trypan blue was able to permeate the cell membrane of dead cells, making them appear deep blue, whereas living cells displayed no blue color.

Locomotor Behavior. DG2807 and DG2847 were prepared in PBS at a concentration of 10 mM. Cockroaches were placed in the modified end of a 15 mL conical tube with the head protruding outward. DG2807, DG2847, or PBS were injected directly into the central complex of the cockroach brain using a Narishige nanoinjector (Narishige, East Meadow, NY) with glass electrode needles. Approximately 200 nL of solution (2 nmoles of each peptide) were injected into each animal. Injection location was determined in a separate batch of cockroaches by injecting a 4% solution of Lucifer yellow in PBS into the brain, and subsequently dissecting out the brain and confirming the location of dye deposition under a fluorescent microscope. Cockroaches were placed in a circular arena (60 cm diameter) approximately 4 hours after injection. Animals were allowed to acclimate to the new environment for 5 minutes, and were subsequently filmed for 10 minutes. Spontaneous locomotion was measured by dividing the arena into 4 quadrants of equal size, and counting the number of times each animal crossed into a different quadrant. All behavioral experiments were performed at room temperature in the afternoon under light conditions.

Venom Peptide Effects on Dopamine Receptors. The effects of venom peptides on dopamine receptors were assayed using the aequorin-based luminescence assay and

insect dopamine receptors described in the previous chapter (Chapter 3). The cockroach D2-like dopamine receptor (PeaD2R) and the *Drosophila* DAMB receptor were expressed in Chinese hamster ovary (CHO-K1) cells, whereas the *Drosophila* dDA1 receptor was expressed in Hi5 cells. Briefly, CHO-K1 cells were grown in monolayers in Dulbecco's Modified Eagle Medium (DMEM)/F-12 containing 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% penicillin/streptomycin, and 1% amphetoricin at 37º C (Invitrogen) and 5% CO_2 atmosphere. Hi5 cells were grown in monolayers in TNMFH insect media (Sigma Aldrich) with 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% amphetoricin at 27º C. Cells were transfected using the same materials and methods described in Chapter 3. Twenty-four hours after transfection, cells were collected and incubated for 2.5 hours with 5 µM of the luminophore coelenterazine h (Anaspec, Fremont, CA). Dopamine-induced luminescence readings were performed immediately after mixing on a Berthold TriStar LB 941 luminometer (Berthold, Oak Ridge, TN) for 20 seconds with a sampling rate of 10 readings/second. Cell culture media was added to wells in place of dopamine as a negative control.

Venom effects on receptor activity was measured in two different ways. The first method involved coapplication of venom peptides DG2807 and DG2847 (individually or in combination) or milked venom (50-55 sting equivalents in a final volume of 100 µL) with dopamine (EC_{80} values, final concentrations of 5 nM, 10 nM, and 1 µM for PeaD2R, DAMB, and dDA1 respectively, Chapter 3) on cells expressing the dopamine receptor. Dopamine and DG2807/DG2847 (final concentration 250 µM) were added by hand to the

wells of a 96-well plate, and approximately 50000 cells were subsequently pumped into each well by the luminometer injector. The resulting luminescence was measured for 20 seconds immediately after mixing. Control cells were handled in the exact same manner, except that cell culture media was added in place of venom/peptides.

The second method involved the incubation of cells expressing PeaD2R (*Drosophila* DAMB and dDA1 were not tested) with venom (50 and 10 sting equivalents in a final volume of 75 µL) or varying concentrations of venom peptides (final concentration range of 333 µM – 0.66 µM) for >30 minutes, and then measuring dopamine-induced luminescence. Approximately 50000 cells were uniformly pumped into the wells of a 96-well plate by the luminometer injector, and venom/peptide solutions were added by hand into the individual wells. After the incubation period, dopamine (final concentration 5 nM) was injected into each well, and the subsequent luminescence was measured for 20 seconds. ATP (which activates intracellular signaling via an endogenous purinergic receptor, final concentration 10 µM) was applied in place of dopamine to determine whether venom peptides selectively affect dopamine receptors.

Results

HPLC-ED Analysis. Analysis of 73 sting equivalents of milked venom with HPLC-ED revealed a large number of distinct peaks. The venom chromatogram contained peaks with retention times that corresponded to tryptophan, dopamine, and homovanillic acid (HVA) (Figure 4-1). The presence of octopamine in the venom could not be determined because there were one or many other coeluting substances at the retention time for

octopamine. In addition, the octopamine peak in both chromatograms had a broad base, suggesting the presence of an additional compound. Peaks with retention times corresponding to L-dihydroxyphenylalanine (L-DOPA), tyrosine, 6-hydroxydopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine, tyramine, and serotonin were absent in the venom chromatogram. There were, however, a number of unidentified peaks in the venom chromatogram. Concentrations of dopamine, tryptophan, and homovanillic acid were estimated to be 105 µM, 60 µM, and 70 µM respectively (based on an average sting volume of 5 nL) (Moore 2003).

Antimicrobial Effects. We examined whether milked venom or venom peptides possess antimicrobial properties. Application of venom/venom peptides had no observable effect on bacterial growth. Gram negative *E. coli* DH5α and Gram positive *B. thuringiensis* grew to confluence on agar plates in the presence of DG2807, DG2847, a mixture of DG2807 and DG2847, and milked venom (71 sting equivalents). The antibiotic carbenicillin effectively prevented bacterial growth and cleared the portion of the plate where it was applied.

Cell viability. The effect of venom and venom peptides on cell viability was measured *in vitro* using mammalian and insect cells. Treatments with 250 µM DG2807 and 250 µM DG2847 (individually or in combination) did not change the level of cell mortality compared to controls in CHO-K1 cells (one-way ANOVA, $p = 0.43$) or in Hi5 cells (Kruskal-Wallis test, $p = 0.14$). Treatments with 63 and 50 sting equivalents of *Ampulex* venom in 200 µL suspensions of CHO-K1 cells and Hi5 cells respectively did not increase cell mortality compared to controls (Figure 4-2). However, statistical analyses

were not performed due to the low number of replicates for each treatment (CHO-K1 n = 1, Hi5 n = 2).

Locomotor effects of peptide injection. Injections of DG2807 and DG2847 (2 nmoles each) into the central complex of the cockroach brain were performed to determine if these peptides have any impact on spontaneous locomotion of cockroaches in an arena. Animals injected with DG2807 (n = 10) displayed significantly lower levels of spontaneous activity, crossing into 6.5 ± 5.9 quadrants in ten minutes, whereas animals injected with DG2847 (n = 10) and sham-injected animals (n = 9) moved considerably more (19.1 ± 8.4 quadrants and 18.3 ± 13.2 quadrants respectively; one-way ANOVA with a *post hoc* Holm-Sidak test, p = 0.013) (Figure 4-3). There was no significant difference between animals injected with DG2847 and sham-injected animals. These results indicate that injection of DG2807 into the brain reduces spontaneous motion in cockroaches, and may be directly involved in venom-induced hypokinesia induction.

Venom effects on dopamine receptors. D2-like dopamine receptors from *Periplaneta americana* (PeaD2R) or *Drosophila melanogaster* (DAMB or dDA1) were expressed in cells (CHO-K1 for PeaD2R and DAMB, Hi5 for dDA1) and subjected to venom and venom peptide application. Coapplication of dopamine (final concentrations of 5 nM, 10 nM, and 1 µM for PeaD2R, DAMB, and dDA1 respectively) and venom peptides (final concentration 250 µM) or venom (50-55 sting equivalents in a final volume of 100 µL) yielded no change in dopamine-induced luminescence in any of the receptors tested (Figures 4-4, 4-5). However, incubation of CHO-K1 cells expressing PeaD2R for >30 minutes with DG2807 or DG2847 and subsequently adding dopamine (final

concentration 5 nM) led to dose-dependent reductions of luminescence (IC_{50}s of 59.5 µM and 181.3 µM respectively). This indicates that these venom peptides, when incubated with cells for >30 minutes, reduced the levels of dopamine-induced luminescence, possibly by disrupting the signaling cascade induced by receptor activation.

To examine whether the suppression of luminescence was restricted to dopamine receptor activation, we applied ATP (final concentration 10 µM) to cells, which mobilizes calcium via a different receptor. Both venom peptides suppressed luminescence when ATP was applied to cells in place of dopamine (IC_{50}s of 106.3 µM and 185.6 µM respectively) (Figure 4-6). This suggests that the venom peptides are not selectively acting on dopamine receptors, but interfering with the signaling cascade shared by both the dopamine and purinergic receptor, ultimately reducing the mobilization of intracellular calcium.

Cell incubation with 50 and 10 sting equivalents of *Ampulex* venom in a volume of 75 µL decreased dopamine-induced luminescence to 19.9 ± 1.6% and 57.5 ± 3.3% respectively of the control value (Figure 4-7). These data agree with the DG2807/DG2847 suppression data, suggesting that reductions of calcium mobilization are primarily due to these venom peptides.

Discussion

Identification and functional determination of bioactive molecules in *Ampulex* venom is critical to determining the mechanisms underlying grooming and hypokinesia induction following envenomation. HPLC-ED analysis suggested the presence of

dopamine in *Ampulex* venom. In addition, the venom chromatogram displays peaks with nearly identical retention times to those of tryptophan and homovanillic acid, suggesting that these compounds may also be found in the venom. GC-MS analysis of milked venom revealed a peak whose mass corresponded with dopamine or octopamine (their integer molecular weights are identical) but could not distinguish which of the two (or both) was present (Weisel-Eichler *et al.* 1999). Our chromatogram showed single peaks at the elution times for dopamine and homovanillic acid (HVA). In mammals, levels of HVA (a dopamine metabolite) have been linked to dopaminergic activity (Bacopoulos *et al.* 1978; Elsworth *et al.* 1987; Wakabayashi *et al.* 1995). This suggests that HVA in the venom was indicative of dopamine's presence, supporting our assertion that dopamine is a venom component. The presence of dopamine in the venom also lends credence to the hypothesis linking compulsive grooming after the head sting to dopamine receptor activation (Weisel-Eichler *et al.* 1999). Our data do not preclude the presence of octopamine in the venom, because of ambiguity surrounding the large peak at the retention time for octopamine. It is possible that octopamine is present within the large peak, but this could not be determined from this experiment. Further analyses are needed to identify the unknown peaks in the chromatogram, and determine whether octopamine is present in *Ampulex* venom.

The presence of HVA implies that there is monoamine oxidase activity in the venom gland of *Ampulex compressa*. In mammals, dopamine is primarily metabolized by monoamine oxidase to DOPAC and HVA. However, in insects, dopamine metabolism occurs primarily through N-acetyl transferase, to form N-acetyl dopamine.

Unfortunately, N-acetyl dopamine was not commercially available at the time of this experiment, so we did not attempt to identify it in the venom. Previous studies have shown monoamine oxidase activity in insects (Tanaka *et al.* 1997; Yellman *et al.* 1997), although activity levels appear to be low compared to N-acetyltransferase activity (Sloley 2004).

Peptides DG2807 and DG2847 are prominent components of milked venom (Moore 2003), but their biological functions have remained obscure. The nucleotide and amino acid sequences of these peptides (which are quite similar between the two) were initially investigated to identify sequence characteristics that may reveal potential functions (Moore 2003). However, BLAST analyses of these two peptides in the Genbank database revealed no similarities to any proteins that have been previously characterized, and no functional properties had been discovered. The lack of homology to known peptides suggests that these peptides are not involved in general housekeeping processes, but have specific functions that are unique to the wasp. We attempted to identify functions for these novel peptides (and milked venom) by testing these compounds in a variety of assays.

Ampulex venom did not demonstrate any antibacterial activity in our experiments. Neither milked venom, nor high concentrations of venom peptides DG2807 and DG2847 (250 μM) were able to prevent the growth of *E. coli* or *B. thuringiensis* on agar plates. This finding was somewhat surprising because antimicrobial peptides have been found in the venoms of different solitary wasps (Konno *et al.* 2001; Konno *et al.* 2006) and are important in the fight against prokaryotic and eukaryotic microorganisms (Otvos 2000;

Zasloff 2002; Kuhn-Nentwig 2003). It is possible that we did not employ sufficient concentrations of peptides to effectively prevent bacterial growth. However, 73 sting equivalents of milked venom did not prevent bacterial growth, suggesting that none of the venom components possessed antimicrobial activity. This also suggests that the peptidergic channel-forming compound in *Ampulex* venom (Gincel *et al.* 2004) is not antimicrobial, but has a different function *in vivo*.

Ampulex venom does not appear to be overtly toxic to cultured cells. Incubation of CHO-K1 cells or Hi5 cells with venom peptides had no observable effect on cell mortality. Milked venom also did not increase cell death, but due to the low number of replicates performed, statistical analyses could not be done to show that venom application had no significant impact. The apparent absence of cytotoxic effects *in vitro* suggests that injection of *Ampulex* venom into the central nervous system of cockroaches does not lead to cell death *in vivo*. If neuronal cell death were the cause of hypokinesia in stung animals, regeneration of damaged areas would need to occur in order for animals to fully recover. There is evidence that axonal regeneration (Patschke *et al.* 2004) and glial regeneration (Smith *et al.* 1987; Smith *et al.* 1991) can occur in insects. However, another study reported that injured axons in the adult CNS of *Drosophila* fail to spontaneously regenerate (Ayaz *et al.* 2008), indicating that neural regeneration does not always occur.

Injections of DG2807 into the central complex of the cockroach brain yielded reductions in spontaneous locomotion compared to sham-injected animals. This suggests that this peptide is directly involved in hypokinesia induction, and this is the first

evidence of a venom component inducing locomotor deficits. Animals injected with DG2847, however, did not display reductions in spontaneous locomotion. This was somewhat surprising because its amino acid sequence is very similar to DG2807 (Moore 2003), yet it does not appear to have the same function when injected into the central complex.

Ampulex venom and venom peptides were tested for antagonistic properties at each dopamine receptor subtype using an aequorin-based luminescence assay that measures receptor activation in real time. Coapplication of venom/venom peptides with dopamine yielded no changes in luminescence, irrespective of dopamine receptor subtype, implying that venom does not immediately inhibit dopamine receptor activation. However, the peptides/venom may simply require longer periods of time to exert any antagonistic effects on the receptors.

The latency between venom injection and hypokinesia induction is around 30 minute *in vivo*, indicating that the venom requires time to exert its physiological effects related to hypokinesia. This latency in behavior suggests that there is spatial separation between the areas of venom injection and downstream targets which directly mediate hypokinesia induction. For example, octopaminergic dorsal unpaired median (DUM) neurons in the thoracic ganglia have lower levels of activity and excitability in stung cockroaches (Rosenberg *et al*. 2006), even though venom injection does not occur in the same location as the DUM neurons. This demonstrates that venom effects in the head ganglia can impact activity elsewhere in the central nervous system, possibly by disrupting descending inputs from the brain. Alternatively, the latency may be due to

changes in gene expression in the brain/SEG which could lead to changes in chemical signaling to the rest of the central nervous system.

Incubation for >30 minutes with individual venom peptides caused a dose-dependent decrease of dopamine-induced luminescence at the cockroach D2-like receptor. Suppression of luminescence was also seen when cells were incubated with 50 and 10 sting equivalents of milked venom, which reinforced the data collected using venom peptides. Luminescence is generated when aequorin is oxidized in response to calcium binding, thus inhibition of the luminescence response indicates that the peptides somehow prevented calcium from interacting with aequorin. One explanation is that the peptides are preventing dopamine from binding to the receptor and triggering intracellular signaling pathways. Inhibition of receptor function would hinder calcium release from the endoplasmic reticulum, thus inhibiting aequorin from producing luminescence. Alternatively, peptide activity may be altering intracellular calcium levels. This could be achieved by changing the permeability of the plasma membrane to calcium, allowing external calcium from the culture media to leak into the cell, or by causing internal stores of calcium to be released. Calcium would then be able to interact with aequorin during the incubation period, oxidizing aequorin prematurely and rendering it inactive, thus eliminating luminescence in response to dopamine receptor activation.

To test whether peptide-induced suppression was specific for dopamine receptors, we applied ATP (which triggers intracellular calcium release via a purinergic receptor) to cells in place of dopamine. Both peptides were able to suppress ATP-induced luminescence in a dose-dependent manner, indicating that these peptides were interfering

with aequorin activation downstream of the receptor, and not specifically inhibiting dopamine receptor function. The IC_{50}s for DG2847 were comparable (181.3 µM and 185.6 µM for dopamine and ATP application respectively), but the IC_{50} for DG2807 in response to ATP binding (106.3 µM) was approximately 2 times larger than the IC_{50} value in response to dopamine binding (59.5 µM). This suggests that DG2807 is better at suppressing dopamine receptor-mediated signaling than ATP receptor-mediated signaling, and may somehow be affecting dopamine binding efficiency.

Reduction of luminescence suggests that the venom peptides are either reducing the amount of intracellular calcium available for signaling, or inducing the removal of aequorin for luminescence generation. The venom peptides could conceivably be acting at any step of the Gq signaling pathway to prevent calcium release from the endoplasmic reticulum (ER). For example, the peptides may act to antagonize the IP_3 receptor on the ER, preventing calcium release. The peptides may also act like thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which depletes intracellular calcium stores, removing calcium available for signaling. Alternatively, the peptides may somehow cause an increase in intracellular calcium levels, rendering aequorin inactive through premature oxidation. Additional experiments are needed in order to determine exactly how these two peptides are causing a depression of calcium-induced luminescence.

Ampulex compressa has evolved a sophisticated chemical arsenal to subdue cockroaches for reproduction. Our experimental evidence suggests that DG2807 and DG2847 interfere with intracellular calcium mobilization. Calcium is an important

second messenger, and interference with normal signal transduction may be the underlying cause behind hypokinesia induction. It is possible that these peptides are disrupting calcium signaling in neurons in the central complex of the brain and SEG of the cockroach, preventing the initiation of locomotor behaviors. Further investigation is needed to determine if these phenomena are correlated.

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Figure 4-1. HPLC-ED chromatogram of 73 sting equivalents of milked venom, compared against a 1 pmole standard mix of 11 electroactive compounds. A mixture of L-dihydroxyphenylalanine (L-DOPA), tyrosine (tyr), 6-hydroxydopamine (6-OHDA), dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), octopamine (OA), tryptophan (TP), homovanillic acid (HVA), dopamine (DA), tyramine (TA), and serotonin (5HT) were fractionated with a C18 reversed-phase analytical column. The venom chromatogram reveals single peaks with the exact retention times of tryptophan, homovanillic acid, and dopamine. Octopamine could not be resolved in the venom chromatogram due to the presence of co-eluting compounds.

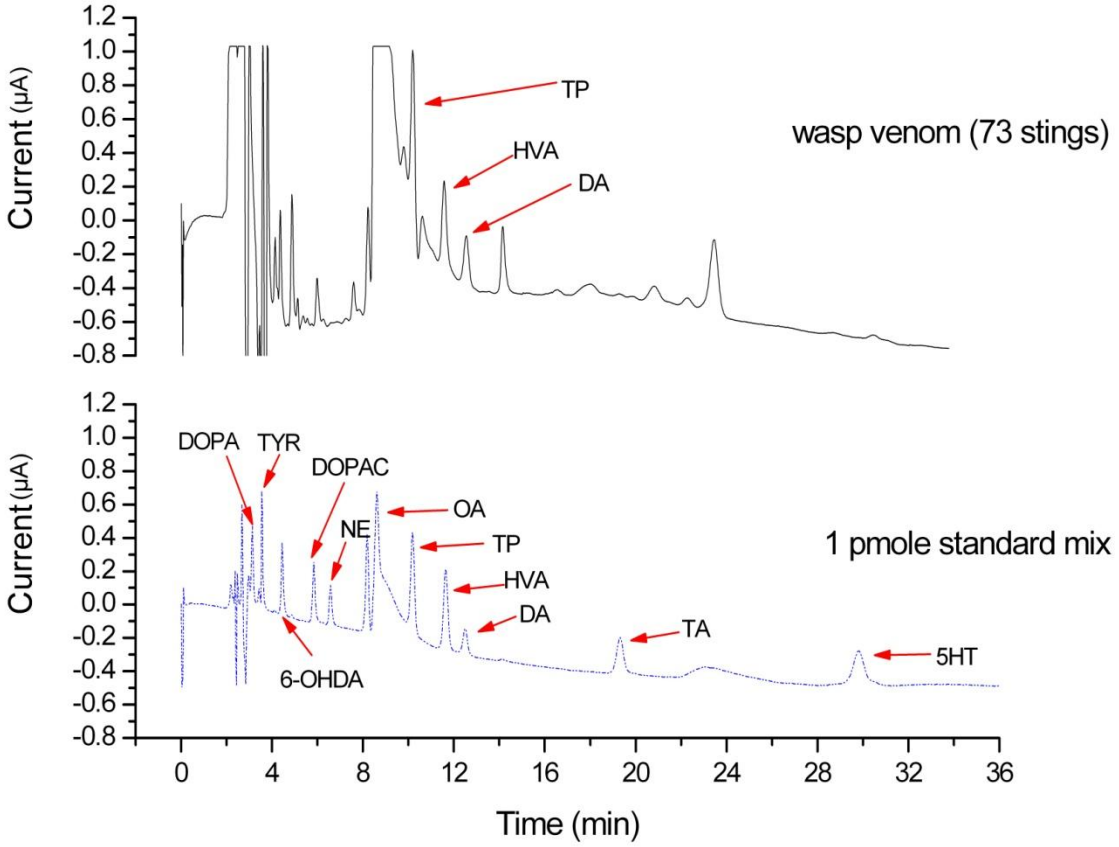
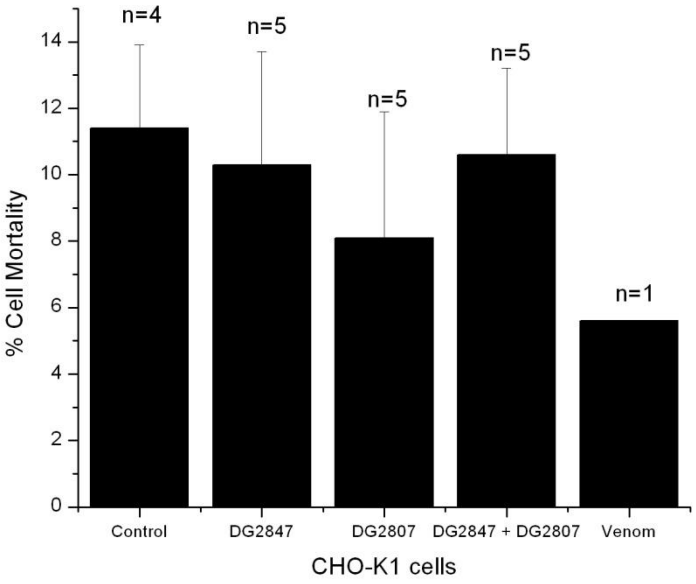

Current (μA)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
TP
HVA
DA
wasp venom (73 stings)
DOPA
TYR
DOPAC
NE
OA
TP
HVA
DA
TA
5HT
6-OHDA
1 pmole standard mix
0
4
8
12
16
20
24
28
32
36
Time (min)

Figure 4-1

Figure 4-2. Viability assay using trypan blue as an indicator of cell death. **A,** CHO-K1 cells were incubated with venom peptides DG2807 and DG2847 (200 μM, individually and in combination) or milked venom (63 sting equivalents in a final volume of 200 μL) for 40 minutes. There were no significant differences between controls and cells treated with the peptides (individually or in combination). One-way ANOVA was performed to assess statistical significance ($p = 0.43$), excluding the venom sample ($n = 1$). **B,** Hi5 cells were incubated with venom peptides (as described in **A**) or milked venom (50 sting equivalents in a final volume of 200 μL). There were no significant differences between cells treated with the peptides and controls (Kruskal-Wallis test, $p = 0.14$). Treatment of cells with venom did not appear to increase cell mortality in either cell type. Statistical analyses were not performed due to the low number of replicates. Error bars represent standard deviations from the mean.

A


n=4
n=5
n=5
n=5
n=1
% Cell Mortality
0
2
4
6
8
10
12
14
Control
DG2847
DG2807
DG2847 + DG2807
Venom
CHO-K1 cells

B

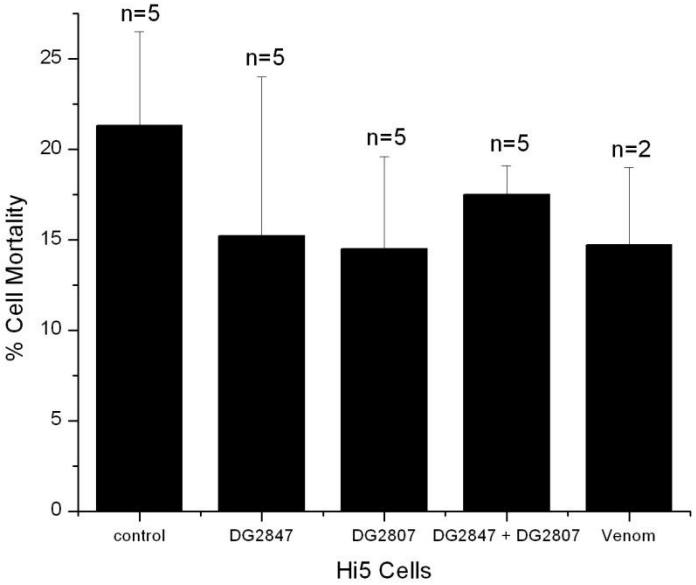

n=5
n=5
n=5
n=5
n=2
% Cell Mortality
0
5
10
15
20
25
control
DG2847
DG2807
DG2847 + DG2807
Venom
Hi5 Cells

Figure 4-2

Figure 4-3. Spontaneous locomotion in cockroaches 4 hours after injection of 2 nmoles of venom peptides DG2847 and DG2807 into the central complex of the brain. DG2807 significantly reduced the amount of spontaneous movement compared to control and DG2847-injected animals ($p = 0.013$). One-way ANOVA with a *post hoc* Holm-Sidak test was performed to assess statistical significance. Error bars represent standard deviations from the mean.

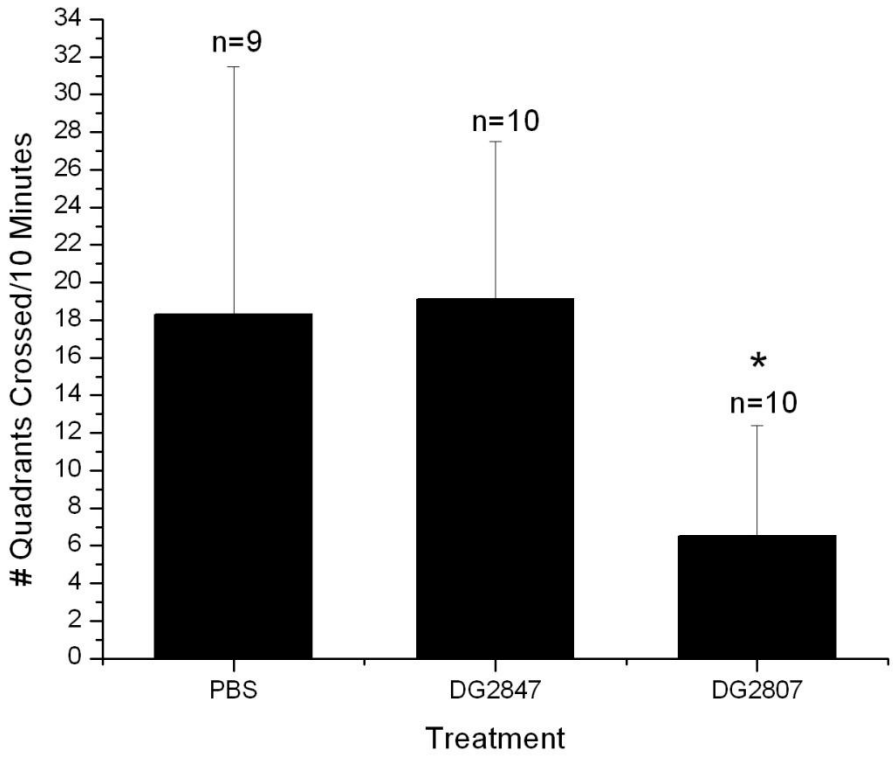

n=9
n=10
*
n=10
# Quadrants Crossed/10 Minutes
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
PBS
DG2847
DG2807
Treatment

Figure 4-3

Figure 4-4. Levels of dopamine-induced luminescence in CHO-K1 cells expressing the cockroach D2-like dopamine receptor (PeaD2R) upon coapplication of dopamine (5 nM) with 250 μM DG2847, 250 μM DG2807, or 55 sting equivalents of milked venom (in a final volume of 100 μL). Luminescence in treated cells (expressed in relative luminescence units, RLU) was normalized to control values (luminescence generated by 5 nM dopamine). Applications of DG2847 or DG2807 had no significant effect on dopamine-induced luminescence signals when applied simultaneously with dopamine ($p = 0.13$). A Kruskal-Wallis test was performed to assess statistical significance.

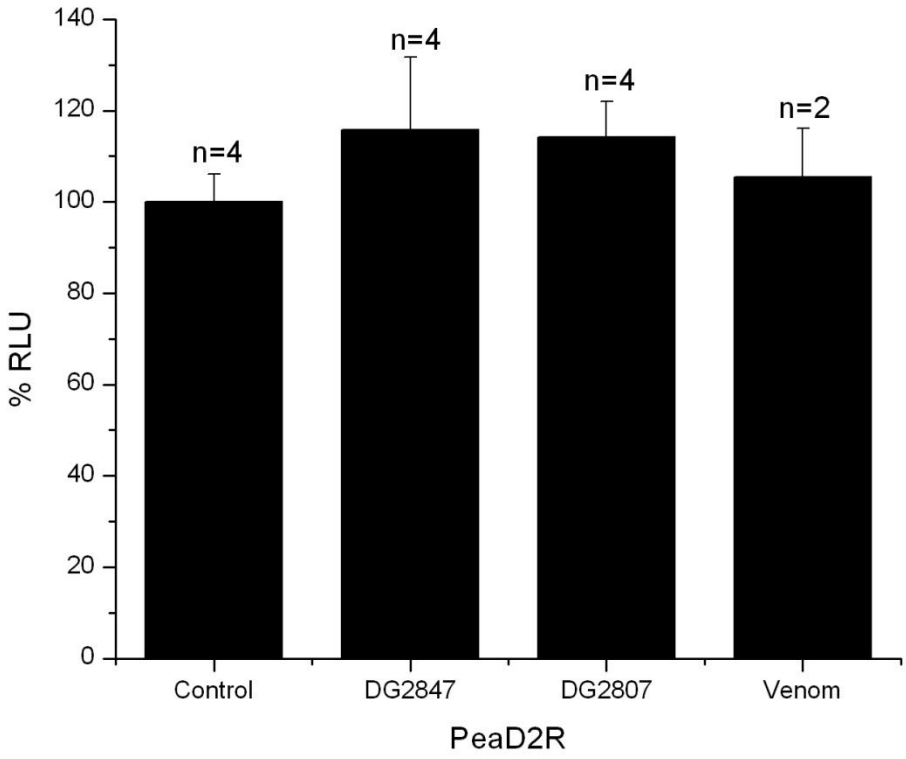
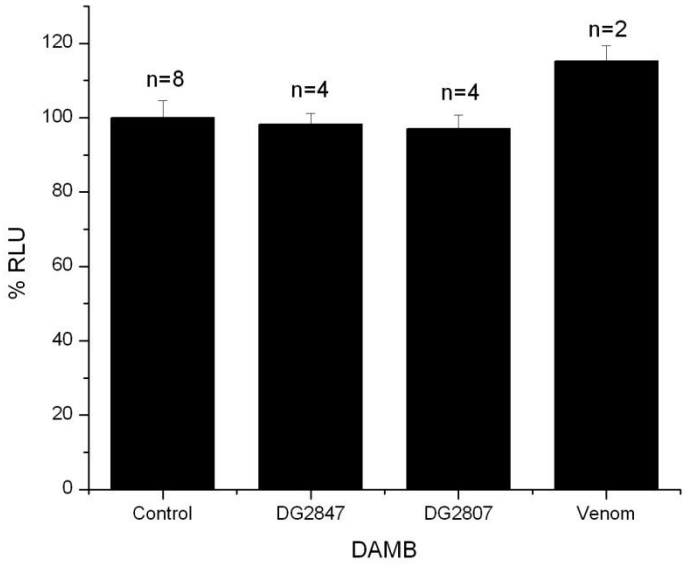

140
120
100
80
60
40
20
0
% RLU
n=4
n=4
n=4
n=2
Control
DG2847
DG2807
Venom
PeaD2R

Figure 4-4

Figure 4-5. A, Levels of dopamine-induced luminescence in CHO-K1 cells expressing *Drosophila* DAMB upon coapplication of dopamine (10 nM) with 250 μM DG2847, 250 μM DG2807, or milked venom (54 sting equivalents in a final volume of 100 μL). Luminescence in treated cells (measured in relative luminescence units, RLU) was normalized to control values (luminescence generated by dopamine alone). Applications of DG2847 or DG2807, or 54 sting equivalents of milked venom did not cause a significant change in luminescence signal ($p = 0.34$). A Kruskal-Wallis test was performed to assess statistical significance. **B,** Levels of dopamine-induced luminescence in Hi5 cells expressing *Drosophila* dDA1 upon coapplication of dopamine (1 μM) with 250 μM DG2807, 250 μM DG2847, or milked venom (50 sting equivalents in a final volume of 100 μL). There were no significant differences between any of the treatments ($p = 0.91$). A one-way ANOVA was performed to assess statistical significance. Error bars represent standard deviations from the mean.

A


n=8
n=4
n=4
n=2
120
100
80
60
40
20
0
% RLU
Control
DG2847
DG2807
Venom
DAMB

B

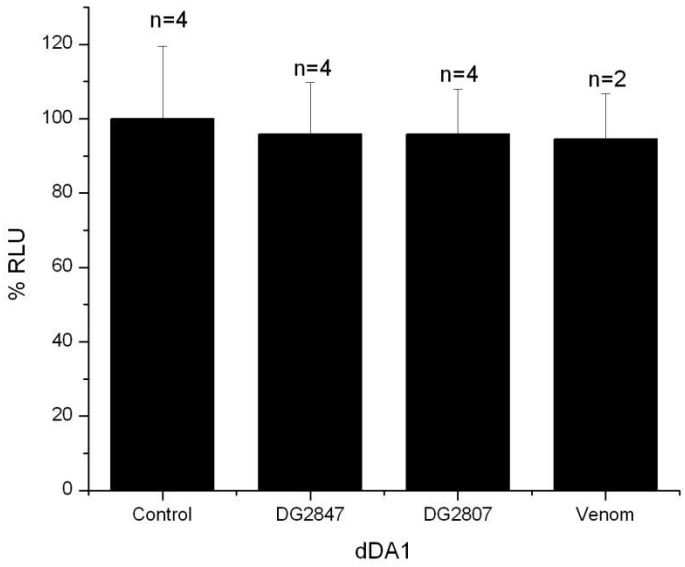
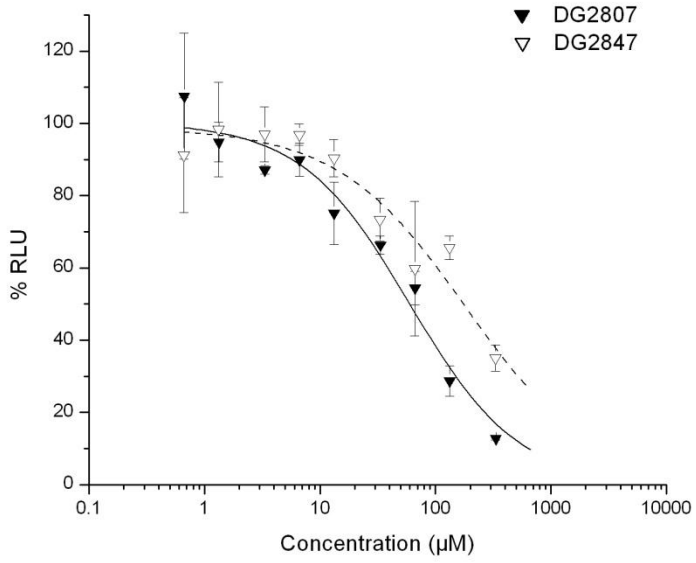

n=4
n=4
n=4
n=2
120
100
80
60
40
20
0
% RLU
Control
DG2847
DG2807
Venom
dDA1

Figure 4-5

Figure 4-6. A, Inhibition curves of venom peptides DG2807 and DG2847 in response to dopamine application. CHO-K1 cells expressing the cockroach D2-like dopamine receptor, PeaD2R, were incubated with individual peptides for >30 minutes prior to dopamine (5 nM) application, and the resulting luminescence was recorded. The IC_{50}s for DG2807 and DG2847 were 59.5 µM and 181.3 µM respectively. **B,** Inhibition curves of DG2807 and DG2847 in response to ATP (10 µM) application. The IC_{50}s for DG2807 and DG2847 were 106.3 µM and 185.6 µM respectively.

A


DG2807
DG2847
% RLU
Concentration (μM)
0
20
40
60
80
100
120
0.1
1
10
100
1000
10000

B

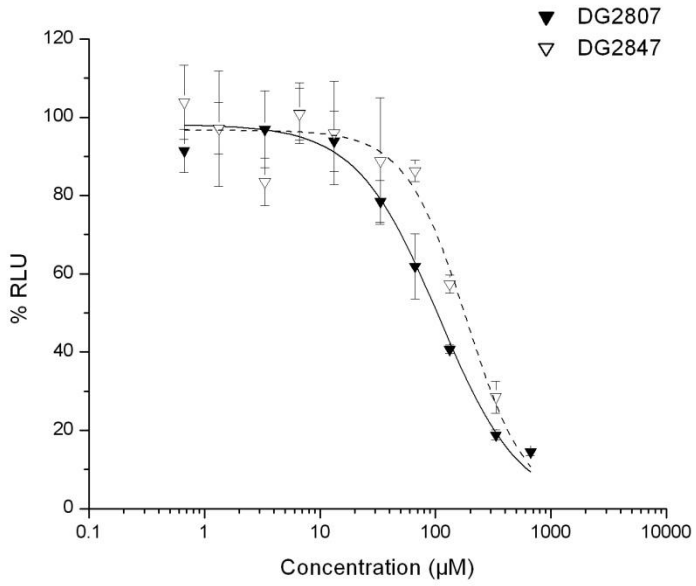

DG2807
DG2847
% RLU
Concentration (μM)
0
20
40
60
80
100
120
0.1
1
10
100
1000
10000

Figure 4-6

Figure 4-7. Inhibition of dopamine-induced luminescence in CHO-K1 cells expressing the cockroach D2-like dopamine receptor in response to milked *Ampulex* venom. Cells were incubated with either 50 or 10 sting equivalents (in a total volume of 75 μL) of milked venom for >30 minutes. Venom application significantly suppressed dopamine-induced luminescence in a dose-dependent fashion ($p = <0.001$). One-way ANOVA with a *post hoc* Holm-Sidak test was performed to assess statistical significance.

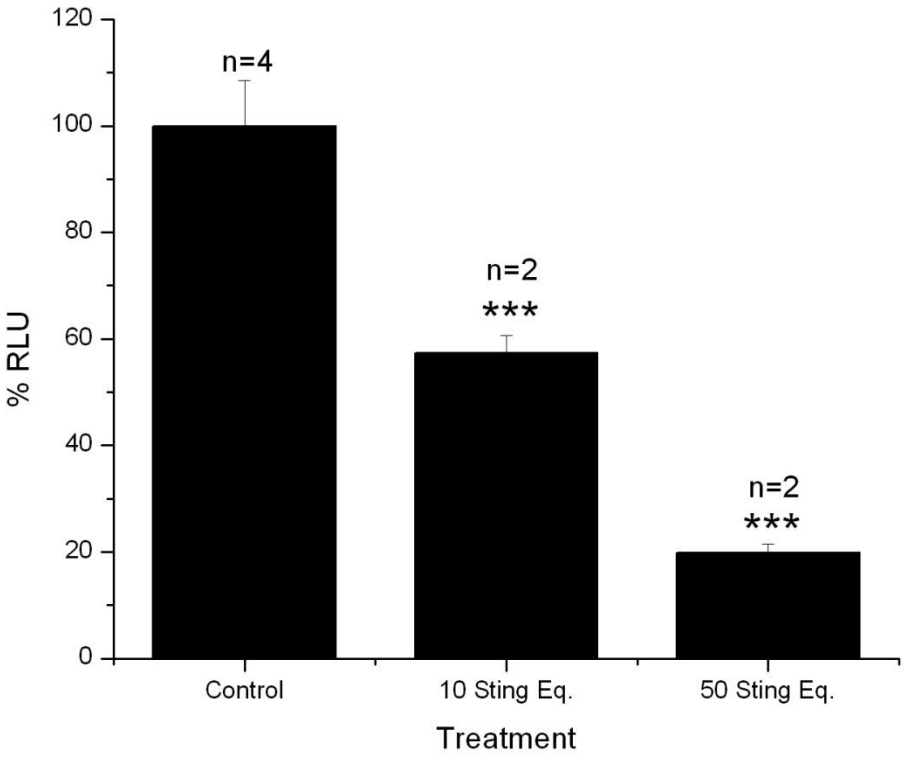

120
100
80
60
40
20
0
% RLU
n=4
n=2
***
n=2
***
Control
10 Sting Eq.
50 Sting Eq.
Treatment

Figure 4-7

Chapter Five

Concluding Remarks

The parasitoid wasp *Ampulex compressa* is a fascinating animal that has evolved to hunt American cockroaches (*Periplaneta americana*) with great precision. Whereas most parasitoid wasps paralyze their prey, *Ampulex compressa* uses venom to induce dramatic behavioral changes in the cockroach which allow the wasp to manipulate the much larger host with virtually no resistance. Stung cockroaches fall into a state of hypokinesia, where cockroaches retain the ability to walk, but cannot escape. Wasp larvae feed on living cockroach tissue until pupation, and adult wasps emerge from the desiccated cockroach exoskeleton approximately six weeks after oviposition.

Because of the unusual mechanism by which *Ampulex compressa* manipulates the cockroach, this wasp's influence has reached beyond the sphere of the entomological and scientific communities, and into the realm of the general public. *Evolve: Venom,* one episode of a documentary series on evolution featured on the History Channel, profiled *Ampulex compressa* in detail. The program Radiolab, on National Public Radio, recently (September 7, 2009) did an episode on parasites, where *Ampulex compressa* was featured. There is even a rock band named Ampulex Compressa. The idea that a parasitoid wasp can use natural chemical weaponry to create "zombie cockroaches" has an appeal that captivates the imagination, and conjures images which could be more appropriate for horror films than scientific journals.

However, the Hollywood nature of this host/parasitoid relationship apart, real scientific questions regarding the neurochemical basis of *Ampulex* envenomation remain. Many advances have been made explaining particular aspects of envenomation. For example, Moore *et al*. (2006) determined that the neuromodulators GABA, β-alanine, and

taurine are responsible for the transient paralysis of the prothoracic legs. Evidence implicates dopamine in the venom as the chemical agent responsible for compulsive grooming after the head sting (Weisel-Eichler *et al.* 1999). However, the cause of the most striking behavioral effect, hypokinesia, has remained a mystery. It has been determined that thoracic dorsal unpaired median (DUM) neurons have reduced excitability and activity in stung cockroaches (Rosenberg *et al.* 2006). Rosenberg *et al.* proposed that the venom prevents descending inputs from the head ganglia from reaching thoracic DUM neurons, indicating that reductions in DUM neuron excitability is a downstream consequence of venom activity in the brain and/or subesophageal ganglion (SEG). The direct biological target of the venom and the venom components responsible for hypokinesia are still unknown.

Research on *Ampulex compressa*, particularly the causes of venom-induced hypokinesia, presented a number of unique challenges. One major obstacle to overcome was the small volumes of venom that could be extracted from the wasp for experimentation. Collecting venom is laborious, and venom output is quite small (the average volume size of a sting equivalent is 5 nL; Moore 2003). In addition, colony maintenance required frequent reproduction, and a balance between milking and reproduction needed to be established, limiting the number of wasps available for milking at any given time.

Direct injection of venom components into cockroach brains *in vivo* was technically challenging, but yielded pieces of informative data. For example, injection of venom peptide DG2807 into the brain reduced levels of spontaneous locomotion,

suggesting that this peptide plays a role in hypokinesia induction. However, this technique was not without its shortcomings. The wasp stings in a precise location in both the brain and SEG of the cockroach, and although the general areas of injection have been determined (Haspel *et al.* 2003), the specific cells that are affected by the venom remain unknown. Any behavioral effect induced through venom-peptide injection only provided clues linking venom components to hypokinesia induction, and provided little direct information regarding specific mechanisms of action. Additionally, there are no data regarding the concentrations of peptides in the venom or the amount of venom injected into the head cavity, thus any dose we applied was simply an educated guess based on reversed-phase HPLC chromatograms of milked venom (Moore 2003). These obstacles and potential roadblocks induced us to search for *in vitro* biochemical assays as alternate methods to investigate hypokinesia.

The lack of an established bioassay for venom-induced hypokinesia was the largest roadblock impeding experimental investigation into the mechanisms of action. A large portion of my graduate work was dedicated to developing methods to determine biochemical correlates to hypokinesia, but the majority of my experiments failed to bridge the gap between behavior and biochemistry, disproving many of our hypotheses. For example, we determined that hypokinesia induction is not due to the depletion of biogenic amines in the head and thoracic ganglia, ruling out the possibility that *Ampulex* venom inhibits amine synthesis or storage. The venom's lack of lytic activity suggests that the venom is not inducing cell death upon injection into the central nervous system

(CNS). In addition, we demonstrated that venom peptides DG2807 and DG2847 do not selectively target dopamine receptors *in vitro*.

The most promising lead for future experiments involves the venom peptide-induced reductions of calcium mobilization. Venom peptides DG2807 and DG2847 were shown to reduce aequorin-based luminescence upon D2-like dopamine receptor and ATP receptor activation in a dose-dependent fashion, suggesting that these compounds do not selectively target the cockroach D2-like dopamine receptor. Similar reductions were seen when cells are incubated with milked venom. Calcium is an important intracellular messenger, and perturbing calcium signaling may lead to changes in gene expression, or potentially interrupt communication between cells. Disrupting calcium signaling in the brain and/or SEG may be responsible for the proposed loss of descending inputs to the thoracic ganglia, leading to reduced excitability in octopaminergic DUM neurons (Rosenberg *et al*. 2006)

Further work is needed to clarify exactly how these peptides are reducing levels of luminescence in our cell assay. These peptides may inhibit one of the many steps that lead to intracellular calcium mobilization. For example, DG2847 and/or DG2807 may antagonize the inositol triphosphate (IP_3) receptor on the endoplasmic reticulum (ER), preventing release of intracellular calcium. Alternatively, these peptides may be pore-forming molecules that facilitate entry of external calcium (and other molecules) into cells. It has been previously reported that a peptidergic channel-forming compound is present in *Ampulex* venom (Gincel *et al.* 2004), but the identity of that compound and its biological functions are not currently known. Perhaps these peptides are involved in

channel formation, or lead to increased permeability of membranes to calcium. In addition, the peptides may induce calcium to leak out of organelles, such as the mitochondrion or endoplasmic reticulum. We will use the aequorin-based luminescence assay to investigate potential mechanisms of intracellular calcium reduction. We are currently using thapsigargin, a compound that selectively causes depletion of calcium in the ER, to determine whether ER calcium stores are depleted in cells exposed to venom or venom peptides. In addition, we will also use a cell-permeable IP_3 receptor agonist to determine whether IP_3 receptors on the ER have been compromised upon exposure to venom peptides.

We are currently in the initial stages of discovering how the venom acts on the CNS. The vast majority of compounds in *Ampulex* venom are currently unidentified, and examinations of these compounds may shed light on other venom functions. Mass spectrometric analyses, such as tandem mass spectrometry (MS/MS) and desorption/ionization on silicon (DIOS), may be useful for identifying polyamines and compounds of low molecular weight. In addition, the production of cDNA libraries from the venom gland and Dufour's gland could potentially identify important peptides and proteins which may contribute to hypokinesia induction. Transcriptome analyses comparing mRNA transcript levels of the CNS of stung and naïve cockroaches using Illumina sequencing technology could also reveal possible changes in gene expression due to venom activity.

It would also be advantageous to investigate venom effects in *Drosophila melanogaster*. The D2-like dopamine receptor is relatively well conserved between

Periplaneta americana and *Drosophila melanogaster* (Chapter 3, Figure 3-3), and it is possible that other important genes are similar between the two species. *Drosophila* is a well-characterized organism with a sequenced genome (Adams *et al*. 2000), and many useful genetic tools available for the study of ethiological and biochemical processes, including Gal4/UAS (Upstream Activating Sequences) systems to control gene expression (Duffy 2002), and mutant/knockout flies to facilitate functional/behavioral studies. These tools would allow us to conduct experiments in fruit flies that would not be practical in cockroaches.

If *Ampulex* venom is acting to disrupt calcium signaling nonselectively, the location of venom injection is critical, because any cells in the vicinity of the venom may be affected. We know that venom is injected into the central complex of the brain and the midline of the SEG (Haspel *et al.* 2003), but we know nothing about the particular neurons affected. Although it appears that the venom does not specifically target the D2-like dopamine receptor, we cannot definitively state that envenomation has no impact on its function. It would be informative to determine the distribution of PeaD2R in the cockroach central nervous system via antibody staining. If the receptor is located in the areas where venom is injected, this would indicate the possibility of the venom inducing dysfunctional dopaminergic signaling.

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